DISTRIBUTION AND EVOLUTION OF ATLANTO-MEDITERRANEAN SPONGES FROM SHALLOW-WATER AND DEEP-SEA CORAL ECOSYSTEMS:

A molecular, morphological and biochemical approach

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DISTRIBUTION AND EVOLUTION OF ATLANTO-MEDITERRANEAN SPONGES FROM SHALLOW-WATER AND DEEP-SEA CORAL ECOSYSTEMS:

A molecular, morphological and biochemical approach

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The Ocean drives the health of this planet (Steve Ross, University of North Carolina, Wilmington)

It is useful because it's beautiful (Antoine de St Exupery)

SUMMARY

Marine sponges (phylum *Porifera*) are among the first branching animal groups (Metazoans), with origins suggested to date from the Pre-cambrian (~635 Myago, Love et al., 2009). They constitute a highly diverse and abundant invertebrate group in hard-bottom benthic communities through tropical, temperate and polar zones, from shallow to deep-sea ecosystems. Sponges have sparked a huge interest in the scientific and pharmaceutical communities due to their **ecological importance** and **production of a diverse range of biologically active metabolites**. Because they are sedentary organism, sponges have developed a wide array of highly effective chemicals against predators, spatial competitors, or as antifouling (see Sarma et al., 1993) which showed a variety of activities (e.g., antibacterial, antifungal, anticoagulant, anti-inflammatory, antimalarial, antiviral, anti-HIV, and anticarcinogenic). In addition, sponges are mostly filter-feeders and play important functional roles in ecosystems (e.g., impacts on substrate, bentho-pelagic coupling, and interactions with other organisms; see Bell, 2008 for a review). Sponges also host extraordinarily dense and diverse microbial communities including bacteria, archaea and fungi, which comprise up to 40% of the sponge volume (Vacelet 1975, Taylor et al., 2007). These microorganisms provide the sponges, along with their filtering capacities, a suite of nutritional and defensive capabilities (Hentschel et al., 2006; Taylor et al., 2007; Van Duyl et al., 2008). It is likely that these symbiotic associations open up new pathways in 1) the production of potent secondary

metabolites and 2) ocean carbon and nitrogen cycles (Hentschel et al., 2006; Taylor et al., 2007). However, sponges represent a group with few morphological phylogenetic informative characters, leading to homoplasies and erroneous classification. Moreover, the intra-and interspecific variation of morphological features is not fully understood yet. In 'keratose' sponges (i.e., taxa which lack a mineral skeleton), and especially in fiberless species (e.g., *Hexadella*), unambiguous species identification is a special challenge. These features make many Porifera prone to cryptic species and taxa boundaries may be confounded, leading to an underestimation of the phylum's biodiversity. These factors may especially impair studies on ecosystem functioning and sponge bioprospection, which require unambiguous species delineation (Erpenbeck and Van Soest, 2007).

Species boundaries and processes that control the distribution of shallow-water sponge species are receiving increasing attention. However, less attention has been paid to sponge species in the deep sea, despite the fact that they are among the most species-rich group in some ecosystems, such as cold-water coral ecosystems (Rogers 1999; Longo et al., 2005). Once believed to be restricted to warm shallow waters in the tropics, coral reefs have been discovered far below the sunlit surface in dark, cold, nutrient-rich waters. Cold-water coral ecosystems are possibly the most three dimensionally complex habitats in the deep sea, and provide niches for many species (Freiwald et al., 2004). However, the reefs are slow growing, particularly vulnerable, and at serious treat (e.g., by bottom trawling). A complex technical deep-sea sampling gear is certainly a major impede for diversity studies of sponges in such remote ecosystems. In addition, the taxonomy of deep-sea sponges is relatively complicated and technically demanding: the extensive presence of small enscrusting sponge species in coral reefs requires high magnification microscopic examination, and easily accessible identification guides (e.g., available for certain shallow-water environments) are lacking for sponges in deeper waters (Van Soest et al., 2007a).

This thesis focused on the species boundaries and phylogenetic relationships of *small encrusting sponge* species in the Mediterranean Sea and along the North East Atlantic margin, from shallow-water to deep-sea coral ecosystems.

In this study, we investigated 1) the distribution and characterization of cold-water coral reef habitats 2) the species boundaries and phylogenetic relationships within the two genera *Hexadella* (Porifera, Verongida) and *Plocamionida* (Porifera, Poecilosclerida), present from shallow-water to deep-sea coral ecosystems in Europe. The principal aims of this study were to explore 1) species distribution and cryptic diversity with molecular markers, 2) the intra and interspecific molecular variation, 3) the use of an integrative approach in sponge species delineation, and 4) the patterns of diversity and connectivity (or isolation) in deep-sea populations.

First, compiling literature (as done in chapter II) on reef habitats and their distribution along the European continental margin highlighted a wealth of studies focusing on cold-water corals in the Mediterranean Sea, the Gulf of Cadiz, and the NE Atlantic slopes from the Irish to the Norwegian margins. However, knowledge about the current coral habitats within a central part of the margin, the Bay of Biscay was actually limited. Studies on the distribution of scleractinian corals in this keystone sector were actually numerous during the nineteenth and the first half of the twentieth century. However they were found to be scattered, sometimes unpublished. In Chapter II, we assembled historical records from as early as 1830 up to 1995, and more recently collected information from two deep-sea cruises, about the occurrence of scleractinian corals in the Bay of Biscay. Published and non-published information allowed to generate a database of 347 records including 34 described species of scleractinians, which nicely documented that the slope of the Bay of Biscay is an important habitat for cold-water coral reefs along the European margin. The data highlighted that coral reefs clustered in 'hotspot' areas along the slope (e.g., the Meriadzek Terrace, the Banc de la Chapelle, the Aquitaine margin, the Cap Breton Canyon, Le Danois Bank and the Cantabrian–Galician margin). These regions were suggested to constitute stepping stone populations for larval dispersal and therefore, the Bay of Biscay was suggested as a semi-continuous habitat for cold-water coral occurrences. These results were also concordant with a worldwide biogeographic study of scleractinian corals which highlighted that one of the largest biogeographic clusters extended from Great Britain to Senegal, and showed some affinities with the Mediterranean Basin (Cairns and Chapman 2001). As such, the Bay of Biscay represented a case study for a better understanding of the distribution of coral and associated fauna along the European margins and the factors that regulate them. This regional study in

the Bay of Biscay stressed (i) a remote and steep slope, (ii) the occurrence of strong bottom currents and water mixing, and (iii) a close association of dead and live scleractinian corals in local areas. Together, these characteristics suggest the presence of suitable environmental settings for the development of filter and suspension feeder colonies in the Bay of Biscay. Although investigations directed to a better understanding about cold-water coral ecosystems in Europe first focused on live corals, co-occurences of dead and live scleractinians were frequently reported along the European margins (e.g., De Mol et al., 2002; Foubert et al., 2005). Coral graveyards (spanning a period of 48 kyr BP) were even found to dominate some areas, such as in the Gulf of Cadiz along the Moroccan margin (Wienberg et al., 2009). This study emphasized that coral rubble and dead fragments can serve as hard substratum for the initial settlement of larva, and may therefore be important for any putative recolonization. All together, these results suggest to focus on both live and dead coral occurrences in any conservation efforts on cold-water coral ecosystems.

With these data at hand, we collected small encrusting sponge species from the genus *Hexadella* (Porifera, Verongida, Ianthellidae) and *Plocamionida* (Porifera, Poecilosclerida, Hymedesmiidae) from shallow to deep-sea coral ecosystems ranging from low to high reef latitudes. Most studies addressing diversity in cold-water corals employed an integral community approach (i.e., with more attention for corals than for other taxa; Jensen and Frederiksen, 1992), or in other cases sponge taxonomists resolved well-known sponge taxa (i.e., megabenthic sponge species; Klitgaard and Tendal 2001, 2004), therefore ignoring lesser-known taxa. Nevertheless, the extensive presence of small sized and morphologically cryptic sponges in cold-water coral reefs was widely noted (Stephens, 1915, 1921; Vacelet, 1969; Boury-Esnault et al., 1994) and was emphasized in recent biodiversity studies in deep-sea coral ecosystems (Van Soest and Lavaleye, 2005, Van Soest et al., 2007a). Although challenging to identify, we expected that the ecological and biochemical importance of these small encrusting sponge species was likely to compete with conspicuous and megabenthic specimens. Species like *Hexadella dedritifera* Topsent, 1913 were suggested to be widely distributed in the deep sea (i.e., from the Mediterranean basin to the Greenland Sea) but such broad geographical range, together with the low dispersal potential usually attributed to Porifera, raised the question of a complex of cryptic species within *H. dedritifera*. Two shallow-water Atlanto-Mediterranean species of the same genus, *H. pruvoti* Topsent, 1896 and *H. racovitzai* Topsent, 1896 showed subtle differences with the deep-sea species.

However, due to their lack of mineral skeleton, verongid identifications are particularly challenging at the intra-ordinal and especially at the species level. In addition, chemical analyses of *Hexadella* species sampled in shallow water and deep sea suggested the production of different secondary metabolites at different depths (Morris and Andersen, 1989). In chapter III, we used the Folmer partition of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the D3–D5 region of the nuclear large ribosomal subunit (28S rDNA) and the second intron of the nuclear ATP-synthetase beta subunit gene (ATPS) to delineate the three European *Hexadella* species, and to investigate cryptic species patterns within the genus. The ATPS marker was tested for the first time for its applicability in sponge species delimitation. Phylogenetic congruence between the independent nuclear and mitochondrial markers illustrated the evolutionary distinctiveness of several lineages within the genus *Hexadella* in both shallow-water and deep-water coral environments and suggested three potentially new cryptic species: the deep-sea species *H. cf. dedritifera* (maybe a junior synonym of *H. pruvoti*), and *Hexadella* sp. (at the moment only known from a deep-sea coral bank in the Ionian Sea) and the shallow-water species *H. cf.pruvoti*. In this study, the phylogenetic resolution in the nuclear ribosomal 28S gene was about 10-fold smaller than for COI and 25-fold smaller compared to ATPS. On the one hand, our analysis therefore showed the usefulness of the ATPS intron marker for taxonomic purposes within the genus *Hexadella*, but on the other hand, due to high evolutionary rates, it was difficult to find adequate alignable outgroup sequences. Therefore, midpoint rooting was used in the phylogenetic reconstruction of the ATPS fragment. In addition, our study illustrated a laborious sequencing step of the ATPS marker (e.g., due to the need to amplify the largest possible fragment of the flanking exons of the ATPS gene in order to allow sequence verification by BLAST search). These issues stressed the difficulty to use the nuclear ATPS markers for phylogenetic studies. Signatures of both genetic differentiation as well as gene flow across large areas in the deep-sea *H. dedritifera* and *H. cf. dedritifera* suggested a complex evolutionary history of deep-sea sponges. These results increased our awareness of the biodiversity of sponges in the deep sea and stressed the need to protect multiple lineages of cold-water coral reefs.

We then searched in chapter IV for phenotypic characters with diagnostic value to differentiate the *Hexadella* cryptic species revealed by molecular markers, and to make these species available to the scientific community. 27 new *Hexadella*-like specimens were

retrieved from four shallow-water Mediterranean locations and described using an integrative approach combining new COI phylogenetic reconstructions, morphological and cytological investigations, biochemical profiling and assessment of natural toxicity in order to identify diagnostic characters for each taxon. Our integrative approach allowed the description of two new species: *H. topsenti* sp. nov. is distinguished from *H. racovitzai* by its colour, the shape of its surface network, a clearly divergent secondary metabolite pattern and significantly different toxicity values. *H. crypta* sp. nov. differs from *H. pruvoti* by a slightly different growth form alive and by coloring distinctively the ethanol when used as a fixative solution. The two species *H. pruvoti* and *H. crypta* also harbor different cells with inclusions. Although *H. pruvoti* and *H. crypta* show slightly distinct metabolic fingerprints, natural toxicity did not allow distinguishing the two species. Our work showed that only the use of a combination of complementary tools can provide relevant descriptions for some problematic taxa, especially when lacking fibers and spicules such as *Hexadella*.

At last in Chapter V, 46 *Plocamionida* specimens were retrieved from seven shallow to deep-water coral locations, from the Gulf of Cadiz to the Norwegian margin. Although the genus has good morphological markers, *Plocamionida* remains a group of sponges that are notoriously difficult to identify. We used a combination of the COI fragment, partial rDNA 28S sequences and morphology to delineate small encrusting *Plocamionida* species, to detect the presence of cryptic species, and to assess phylogenetic relationships within this group. Comparing the limited number of morphological characters against a robust and comprehensive phylogenetic (DNA) tree proved to be a fruitful approach for integrating the strengths of morphological data with those of sequence data. Although the description of new species can be a laborious and long process for some taxa (i.e., a whole revision of the genus should be performed), our study showed that a comparison of morphological data with (congruent) molecular phylogenies can help the taxonomist to better understand the intra- and interspecific character variation and to resolve the current taxonomic difficulties in some taxa. The potentially new *Plocamionida* species will be targeted in further sampling and detailed phenotypic diagnostic analyses in order to support the observed molecular differences. Following the identification of divergent lineages within *Plocamionida* sp, we afforded a phylogeographic study within the most widespread *Plocamionida microcionides* species. Although these data suggest the connection between populations of cold-water coral

associated species along the NE Atlantic margin, a more extensive sampling would be necessary to support these tentative conclusions. These preliminary data are hence provided as an appendix to Chapter V (Addendum III).

In conclusion, the diversity studies of small encrusting sponge species *Hexadella* and *Plocamionida* in shallow to bathyal ecosystems led to the discovery of a large amount of new cryptic species, especially in cold-water coral ecosystems. This might be expected in remote environments such as the deep sea, as many species 'new to science', from all phyla, are recurrently reported. But the high genetic and species diversity found in some cold-water coral locations suggested these reefs as diversity hotspots for the phylum Porifera. Allopatric and sympatric cases of cryptic species might be explained by both historical biogeographic factors (i.e., related to glacial and interglacial cycles), as well as high diversity of small niches in complex coral habitats (i.e., live coral intricate with coral rubbles and associated species are suggested as important sources of biogenic heterogeneity). In addition, these studies stressed that both mitochondrial COI and nuclear markers 28S showed distinct levels of genetic variation between *Hexadella*, *Plocamionida*, and other sponge species. These results further emphasize the need to obtain sufficient data on intra- and interspecific variation of molecular markers to delineate species. At last, an integrative approach was shown to outperform traditional approaches to investigate species boundaries, in the phylum Porifera.

CHAPTER I

General Introduction

CHAPTER I

The Importance of 'Diversity' Units: Biodiversity and Ecosystem Functioning

Life is the most remarkable feature of Earth, and the most remarkable feature of life is its diversity. Biological diversity usually refers to all biological differences and is typically defined as a hierarchy: it ranges from diversity of ecosystems and habitats (*habitat diversity*), to diversity of species within an ecosystem or habitat (*species diversity*) and to diversity of individuals within a species (*genetic diversity*; Noss 1990; Chapin et al., 2000). Understanding the different patterns of biological diversity, the underlying processes that control the origin, distribution and evolution of organisms and how these relate to contemporary and historical factors are crucial to construct future management plans. That knowledge can light up essential ecological and evolutionary processes and lead to sound conservation policies. This is particularly relevant since **biodiversity and functioning of ecosystems are intertwined**, as demonstrated by a number of terrestrial studies (Chapin et al., 1997, 1998, 2000; Tilman, 2000; Loreau et al., 2001; Hooper et al., 2005; Cardinale et al., 2006). The importance of marine benthic biodiversity for ecosystem functioning has also been increasingly shown during the past two decades (Steele, 1991; Micheli and Halpern, 2005). For instance, some recent studies emphasize that species from the phylum Nematoda (De Mesel et al., 2003; Danovaro et al., 2008) and Porifera (see Bell, 2008) can significantly impact bentho-pelagic ecosystems. Generally, functions of an ecosystem are related to productivity (i.e., biomass accumulation as a measure of energy assimilation; MacArthur, 1955) and stability together with ecosystem resilience and resistance (Johnson et al., 1996). Ecosystem processes can be summarized as the rates of energy and nutrient flow between biotic and abiotic compartments, such as biomass production, nutrient regeneration, nutrient production, transport, or organic matter decomposition and transfer to higher trophic levels, as well as nutrient dynamics (e.g., nitrogen mineralization rate, nitrate leaching; Chapin, 1997).

Species diversity has functional significance because natural communities differ greatly in the number and kind of species performing different ecological functions. All together, the number of species present (species richness), their relative abundances (species evenness), the kind of species present (species composition), the interactions among species (non-additive effects), and the temporal and spatial variation in these characteristics determine the expression of traits that influence ecosystem processes. The relationship between species

richness and ecosystem functioning is theoretically simple when all species have complementary niches (i.e., they contribute substantially and in unique ways to a given process; Chapin et al., 2000). However, this association has been substantially debated (Johnson et al., 1996), especially in species-rich ecosystems or when one or a few species have strong ecosystem effects (Chapin et al., 2000). Some species can indeed have strong direct or indirect (by altering abiotic conditions) effects on ecosystem processes (e.g., grass invasion can increase fire frequency which destroys native trees in Hawaii; D'Antonio and Vitousek 1992). It also seems that environmental change influences more frequently the relative abundance of species than the simple presence or absence of species (Chapin et al., 2000). In addition, species interactions (competition, mutualism and trophic relations), rather than species number alone, can have strong ecosystem effects and are a general feature of ecosystem functioning (Chapin et al., 2000). In most cases, ecosystem processes are non-additive functions of the expression of species traits. Specific species interactions determine the stability of ecological systems (Johnson et al., 1996) and further determine ecosystem characteristics (e.g., highly integrated communities such as consortia of marine microorganisms mediating anaerobic oxidation of methane, Boetius et al., 2000). And because of such specificity, it is highly likely that loss (or invasion) of a particular species will have cascading effects on the remaining system. The nature or timing of species interactions are also of great concern, and altered by many global changes.

Although many studies focus on species diversity, **genetic diversity** is also known as functionally important. For example, the viability of crops depends on the preservation of high genetic diversity, which can improve resistance to emerging and evolving diseases and pests (Vanderplank, 1984), reducing the risk of catastrophic losses caused by epidemics. Then, the phenotypic distinctions, bounded to a particular genotype and determining functional uniqueness may not conform simply to distinctions at the species level, since genetic diversity could be of equally great importance (Johnson et al., 1996). Therefore, genetic diversity is together with species and ecosystem diversity one of the forms of biodiversity recognized by the IUCN (International Union for Conservation of Nature) as requiring conservation (McNeely et al., 1990).

Reliable knowledge about the relationship between diversity and ecosystem functioning and about the functional role of species diversity in ecosystems can only be

achieved when 'diversity units' are correctly identified (Johnson et al., 1996). Although it is now fully recognized that the discrimination of species underpins all biological studies, species delineation is complex for some groups of organisms with a relative paucity of quantifiable morphological characters or with malleable overall forms. Some organisms are notoriously difficult to identify because the intra- and interspecific character variation is not well understood (see chapters herein), and has given rise to disagreements between experts. In addition, despite its importance, biodiversity became only relatively recently a hot topic of research in some remote environments, such as the deep sea. In the marine realm, the first Census of Marine Life took place only during the last decade (2000-2010, http://www.coml.org).

How biodiversity is structured and how it is related to ecosystem functioning are scientific questions of considerable fundamental interest. But these are also burning questions in terms of human ecosystem management. We live at a time of rapid human-induced environmental and climate change, and simultaneously, of accelerating rate of habitat losses and species extinctions. These global environmental changes (global biogeochemical cycles, land transformation and mobility of biota) alter biodiversity which in turn alters ecosystem processes and the resilience and resistance of ecosystems to environmental changes (Fig.1). The loss of local biodiversity due to the absence of sustainable management practices has the potential to impair ecosystem functioning and many important services to humankind (Chapin et al., 1997).

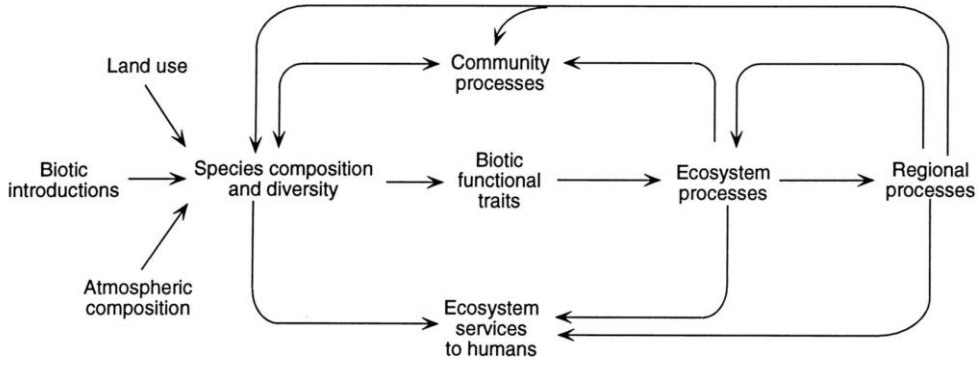


Figure 1. Linkages involving species composition and diversity and ecosystem processes (from Chapin et al., 1997).

Less obvious but also important to societies, is the fact that biodiversity and its links to ecosystem properties have cultural, intellectual, aesthetic or moral, ethical or spiritual values. These **intrinsic values** of biodiversity are based on the idea that humans are an intrinsic part of nature. Wilson (1984) suggested an instinctive tendency [in human beings] to center on life and lifelike process, which he named '*biophilia'*, implying that such human aspiration to remain surrounded by biodiversity and to manage nature could have been driven by the process of natural selection. Expressions of aesthetic and moral values of nature and its biodiversity are actually found in all civilizations and filling major parts of Arts. Poetry and literature, music, dance, painting and sculpture show how human beings have developed and cultured a deep attachment with nature and its components, such as forest, ocean, or animals. The worship of particular trees and plants (e.g., Neem, Banyan, Tulsi) or animals which are considered as vahanas (i.e., means of transport) of different gods and goddesses by Hindu societies are important examples (e.g., cows and their offspring or elephants which are seen as form of Lord Ganesh). These cultural values of biodiversity protect plants and animals in many parts of the world.

However the protection of biodiversity is not only an ethical issue, it is also timely from a socio-economic perspective (Danovaro et al., 2009). Biodiversity and the natural environment provide services, which benefit the economy (i.e., goods production and exchange) and constitute the base of the so-called **anthropocentric theory**. Food, fuel and oxygen are the most important example goods obtained from nature. But other goods include fibre, pharmaceuticals and medicines that are derived from naturally existing resources. Some organisms have proven to be incredibly valuable to humans (i.e., penicillin, a group of antibiotics derived from *Penicillium* fungi) and millions of others remain to be tested chemically, leaving many open opportunities for discovery of new compounds. The recreational and aesthetic benefits of nature, which have allowed camping, birding and diving to remain extremely popular are also a great source of economic wealth and considered anthropocentric.

Recognizing Diversity Units

Linnaeus and other taxonomists referred to what Ernst Mayr (1963) later called a 'typological ' or 'Essentialist' species concept. In this philosophy, species conveyed the idea (which stemmed from Plato) of a constant class of objects in which variation is an accidental

imperfection, and only essence (the‘eidos’) matters. However, the importance of variation within and among populations, together with the acceptance of Darwin’s vision that all characteristics can vary and evolve, led to the rejection of this essentialist species concept. Classical taxonomists classified individuals as members of a species based on a set of shared diagnostic morphological characters that differentiated them from other such morphologically defined groups. Nevertheless a great number of cryptic species (i.e., species that are difficult or impossible to differentiate based on morphological features but which are genetically distinct; Knowlton, 1993) were detected using other markers (see below). Moreover, in some groups of organisms, a single genotype may produce more than one form of morphology, physiological state, and/or behavior in response to environmental parameters (i.e., phenotypic plasticity, West-Eberhard, 1989). Therefore the morphological species concept became less used (which does not mean, however, that morphology cannot be used to delineate species, as we shall see). Numerical taxonomy (or phenetics, see below) shares the concept of a morphological species and differs from classical taxonomy mainly in its approach (the use of as many features as possible, rather than a few characters that the taxonomist subjectively felt ‘important’), and in its emphasis on species as clusters derived from degrees of overall similarity (Sneath and Sokal, 1973). The central biologically based concept of Mayr (1963) defines species as groups of actually or potentially interbreeding populations isolated from other such groups. Under this concept, new species are formed when they are reproductively isolated. However, mechanisms of reproductive isolation differ among taxa, therefore the Biological Species Concept (BSC) offers no universal standard to delimit species. Specific knowledge on each taxa remains needed to discriminate species. Among the many other species concept that have been proposed, the most popular current competitor with the BSC is the phylogenetic species concept (PSC). The PSC emphasizes on the phylogenetic history of organisms and defines species in terms of monophyly (i.e., in a monophyletic group, all its members are believed to stem from a single common ancestor). For this concept and other cited above, the drastic improvements of genetic tools in the last decades have allowed a new phase in the study of biological processes and species discovery.

Genetic studies started with the theories of heredity of Mendel, based on his experiments with the garden pea *Pisum sativum* (1865). But the full significance of his work was only recognized in the early 1920s and early 1930s in relation to evolutionary theory. Fisher (1930) applied Mendelian theories to populations. However, major advance in our

understanding of genetic variation in natural populations began in the mid-1960s with the advent of molecular biology. First, protein electrophoresis (based on the relationship between DNA base pair sequences and amino acid sequences) allowed assessing genetic variation in a wide variety of species (Singh et al., 1976). Then, a real methodological revolution arose with the introduction of the Sanger dideoxy sequencing method in the late 1970s (Sanger et al., 1977) and the polymerase chain reaction (PCR) in the mid 1980s (Mullis and Faloona, 1987), allowing to obtain DNA sequence data.

Genes are the physical units of heredity that are transmitted from generation to generation during reproduction. They reside on a DNA molecule (or RNA in the case of certain viruses), the sequence of which is associated with regulatory regions, coding regions, and/or other functional sequence regions. The different variants of the gene - *alleles*- are the genetic make-up of a species. Genetic variation is the raw material of evolution and the ultimate source of all genetic variation are *mutations*. These include changes in the DNA sequence (substitution, insertion, deletion) from a single nucleotide to larger fragments, but also chromosomal rearrangements, recombination and lateral gene transfer. Changes in allele frequency over one generation are generally not affected by any specific mutation, which is a rare event. However, the action of three forces can significantly alter the frequency of alleles: selection, random genetic drift and migration (Hartl, 2000). Natural selection is a directional process, which changes the frequency of alleles within a population via differential survival and reproductive success of individual organisms. The ones who have a greater "fitness" (i.e., with well-adapted phenotypes) will pass a greater proportion of their genes to the next generation. This was already proposed by Darwin in 1859 in 'The origin of species' as the driving force of evolution. Another directional process is migration, which involves the introduction (immigration) or loss (emigration) of individuals in a population, and therefore increases the exchange or transfer of genes and alleles (i.e., gene flow). The process of migration followed by successful reproduction of the new migrants has the tendency to homogenize disparity in allele frequencies between the source and the sink population. At last, a completely random process affecting all populations and especially the smaller ones is genetic drift. It refers to fluctuations in allele frequency as a result of random loss of alleles because they are not passed to the next generation. Special cases of genetic drift are bottlenecks and founder events; the founding of a new population by a small number of

individuals (i.e., a severe bottleneck in population size) will cause rapid changes in allele frequency and loss of genetic variation.

These microevolutionary genetic processes lead to differential allele frequencies between populations, and ultimately form the basis of species formation (Stearns and Hoekstra, 2000). In the basic model of allopatric speciation, a large and continuous population is split up into smaller ones by extrinsic barriers. Genetic exchange between these separated demographic units then stops, so that genetic differences between them build up. Ultimately, genetic divergence leads to intrinsic barriers for reproduction (Palumbi, 1994). However also more controversial modes of speciation (i.e., sympatric speciation) which occur under selection pressure should also be considered. Whereas population genetics (microevolutionary analysis) and phylogenetic biology (macroevolution) were formerly separated disciplines, phylogeography forced a bridge between both research areas (Avise, 2009). The word phylogeography was coined in 1987 and emerged as a research field seeking to interpret the historical evolutionary processes which have determined the contemporary geographic distribution of genealogical lineages within and among species (Avise, 2000). The significance of genetic variation for biodiversity evaluation has been increasingly recognized (Moritz and Faith, 1998).

In this book, entities satisfying the criterion of reciprocal monophyly for mtDNA alleles and showing divergence at nuclear loci were eligible for recognition (Evolutionary Significant Units, species). Such definition has the advantage of avoiding the issue of 'how much divergence is enough to delineate species' and allows to follow the primary line of reasoning of cladistics (i.e., to define monophyletic groups as evolutionary entities). However in some circumstances it may seem overly restrictive and as with all evolutionary properties of species, the definition of an ESU needs to be applied with common sense. In addition, if the entities differed in a number of other features and the characters were correlated, our suspicion that they are species grows stronger as the number of such distinguishing features increased.

CHAPTER I

Phylogeny

Speciation creates a branching tree of relationships that express evolutionary history. The goal of systematics is to **discover the structure of that tree**, to infer the relationships of all livings things and thereby understand the history of life since its origin. The question is how to get the best estimation of this idealized tree?

By using a comparative approach, biological information can be organized into a meaningful estimation of the evolutionary relationships among lineages of organisms, i.e., a phylogeny. Traditionally, phylogenetic trees used mainly physical or morphological characters (e.g,. size, color, etc.), while modern phylogeny adds genetic information, mainly DNA and protein sequences. The increased number of characters available has been seen as the greatest advantage of molecular data (Hillis, 1987). The phylogenetic tree can therefore provide a natural and meaningful way to order data, and contains an enormous amount of evolutionary information within its branches (Page and Holmes, 1998).

To infer molecular phylogenies, and to convert sequence information into an evolutionary tree, a plethora of methods have been described. Generally trees are reconstructed using different reconstructing methods (Exhaustive Search, Branch and Bound Search, Heuristic Search, Markov-Chain Monte Carlo) and in combination with different optimality criteria. When data sets become too large to use exact searching methods (Branch and Bound or Exhaustive Search), it is necessary to use heuristic methods (i.e., approximate methods that try to find optimal solutions). However, using heuristic methods do not warrant that the most parsimonious tree will be found. These methods employ hill-climbing algorithms to progressively come up to the best tree. Nevertheless, there can be "tree islands" of suboptimal solutions, and the analysis can become entrapped in these local optima. Thus, to attempt that tree space has been adequately explored, several complex additional algortithms are available (branch-swapping algorithms, e.g., tree bisection/ reconnection (TBR)) or multiple repetitions of the analyses with different starting points are required.

The optimality criteria are divided by the way they handle data, or alternatively by the approach they take when building tree. A division based on the way the data is regarded,

separate the distance methods, which convert aligned sequences into a distance matrix and deduct the tree from these distances, from discrete methods which regard shared character states (i.e., apomorphies). These two philosophies of building phylogenetic trees are also referred to as phenetics and cladistics. Phenetic methods construct trees (e.g., phenograms, based on overall similarity) by considering the current states of characters without regard to the evolutionary history that brought the species to their current phenotypes. Cladistics methods construct trees relying on assumptions about ancestral relationships as well as current data. Cladistic methods comprise e.g., maximum parsimony, maximum likelihood and Bayesian methods. Parsimony methods assume that among the various phylogenetic hypotheses that are formulated for a group of taxa, the best phylogenetic hypothesis is the one that requires a minimum of evolutionary changes (i.e., the simpler solution is the best). Maximum likelihood aims to reconstruct the evolution across sites with substitution models to assess the probability of particular mutations. The method then evaluates the likelihood, for the candidate tree topology that the tree will give rise to the observed datamatrix (e.g., sequences) given the evolutionary model of sequence evolution. In other words, the tree with the highest likelihood is the best hypothesis. Maximum likelihood is computationally demanding to perform on many sequences. Recent advances (RAxML, Stamatakis 2006) reduced this computational time to a linear increase with sequences number, thus allowing very large phylogenetic sampling (e.g., Dunn et al., 2008). Bayesian methods are closely related to the maximum likelihood methods. However they allow for the consideration of a priory distribution of many parameters (i.e., the topology, the branch lengths, the four stationary frequencies of the nucleotides, the six different nucleotide substitution rates, the proportion of invariable sites, and the shape parameter of the gamma distribution of rate variation, etc) out of which they produce in combination with the likelihood of the data a posterior distribution for that parameter (i.e., posterior probability of a tree). A very useful numerical method, the Markov Chain Monte Carlo tree reconstruction (MCMC, allowing the posterior probabilty of a tree to be approximated) has revolutionized Bayesian inference, with recent applications to Bayesian phylogenetic inference.

There is a fundamental difference between the true evolutionary pathways of a group of species (i.e., species tree) and a single gene tree. Gene tree and species tree can have incongruent topology or branch lengths, due to gene duplication (resulting in paralogous genes), incomplete lineage sorting and horizontal gene transfer (Page and Charleston, 1997).

Therefore, the use of different markers to construct and compare trees is considered as a more robust method than single gene trees. In addition, intraspecific gene evolution (i.e., reticulate evolution) often violates the assumptions of these 'traditional' phylogenetic reconstruction methods (i.e., bifurcating trees), leading to a poor estimate of intraspecific genealogies. Several population evolutionary patterns (e.g., low sequence divergence, descendant coexisting with extant ancestors, multifurcations, reticulation, large sample sizes) indeed produce reticulate relationships. The non-recombining characteristic of the 'blessedly celibate' haplotypes in plastid genomes (Dawkins, 1995) have allowed mitochondrial DNA to reconstruct branched and non-reticulated trees (i.e., gene genealogies or coalescent). However for nuclear genes, new phylogenetic reconstruction methods such as networking approaches, which take into account these population-level phenomena, may be a more appropriate representation of intraspecific variation (Posada and Crandall, 2001).

The incorporation of molecular techniques into the field of systematics has allowed to detect many cryptic species (or sibling species) and to investigate the phylogenetic relationships among lineages of organisms, or groups of organisms. The study of molecular signatures present in contemporary populations is now widely used for establishing baselines of species diversity and the management of resources. An increasing number of studies are indeed focusing on species boundaries, distribution and on processes that control species ranges in habitats or ecosystems**.** However, studies from 'remote' environments such as deep-sea ecosystems are still very scarce.

The Deep Sea: the Last Frontier on Earth

More than two-thirds of the Earth's surface is covered by ocean. The depth of the oceans ranges from shallow bays and estuaries, to a depth of about 11,000 meters at the deepest point, the Mariana Trench (located in the Pacific Ocean). Taking into account the vertical extent of the deep sea, with an average depth of about 4,000 meters, it represents 90 % of the volume of the world's oceans. The deep sea is hence the largest ecosystem on the planet. Gage and Tyler (1991), in the preface of their famous book on Deep-sea Biology, stated:

We may therefore, with some justification, speak of the deep-sea bottom as constituting the most typical environment, and its inhabitants as the typical lifeforms.

However, the vast deep sea remains largely unexplored (Levin et al., 2001), and less than 0.01% of the deep-sea floor has been sampled and specifically studied (Ramirez-Llodra et al., 2010).

During the 19th century, the prevailing opinion was that the dark water column was impenetrable and that the deep sea, loosely defined as waters below 200 meters, was characterized by the hardly hospitable physical conditions of cold temperatures, darkness, and increased pressure. The deep sea was sparking the imagination of the greatest authors, poets and artists. The 25th of July 1865, the French writer George Sand who was charmed by the last two books of Jules Verne (Voyage in the center of Earth and From Earth to Moon), wrote his friend:

I hope that you will soon escort us into the deep sea and that your performers will evolve in these diving instruments that only your knowledge and your imagination can create.

Jules Verne provided with the adventure of the captain Nemo in his novel "Twenty Thousand Leagues Under the Seas" a fantastic journey in shallow to deep waters, for the delight of young to old dreamers' generations, and didn't deceive his contemporaneous colleague.

But the curiosity and passion of Man changed this fantasy. In 1818, Sir John Ross recorded for the first time deep-sea fauna at 1,600 m (Menzies et al., 1973). At that time, this discovery remained unknown and Forbes (1844) developed the "Azoic Theory" for depths below 600 m, after recording less species with increasing depths down to 420 m on board of the H.M.S. Beacon, 1841-1842. However, the hypothesis was being discussed, encouraging investigations and in the years that followed, evidence of life in the deep sea was repeatedly reported. The founder of modern oceanography is considered to be C.W.Thomson who in 1872 collected specimens down to a depth of 5,500 m during an expedition of more than three years on board of the H.M.S Challenger, showing that the deep ocean is not devoid of life, as previously thought (Menzies et al., 1973).

From then on, deep-sea exploration has been achieved at increasing pace, leading to new taxonomic discoveries and to the development of new technologies to explore the depths.

The Second World War gave a significant impulse to oceanographic research, as complex technical survey tools, such as sonographs, current measurement tools, high resolution seismic profiles, and side-scan sonar imagery were designed. It allowed to map the seabed extensively, and has lead to new exploration possibilities (Koslow, 2007). In the 1960s and 1970s, deep-sea research has moved from qualitative to quantitative investigations, thanks to the development of sampling gears and the introduction of statistical tools for sample comparison (Sanders, 1968). But the advent of extremely specialized devices, such as Remote Operated Vehicles (ROVs) or vehicles able to work and make measurements on the seabed autonomously, without being bound to the ship above (e.g., Autonomous Underwater vehicle (AUVs) or submersibles) revolutionized the conquest of the ocean, allowing exploring and experimenting in situ. These deep-sea vehicles often provide spectacular pictures and video-footage to illustrate the discovery of unseen creatures and habitats which are beyond our imagination and serve to inspire the public and scientific communities (e.g., hydrothermal vents at the Galápagos Rift in the Pacific in 1977; cold seeps in the deep Gulf of Mexico in 1984; a new class of hydrothermal vents located 15 km from the Mid Atlantic Ridge, the Lost City in 2000).

These discoveries shed light on extraordinary, unexpected life thriving in these habitats, therefore changing our understanding of planet Earth and life on it. But the significant contribution of the deep sea to very fundamental biogeochemical cycles such as the carbon and nutrients (e.g., phosphorus, nitrogen, sulphur, etc) cycle that support all life (Gage and Tyler 1991; Dell' Anno and Corinaldesi, 2004; Dell' Anno and Danovaro, 2005; Buesseler et al., 2007) has a greater significance to humankind and our planet. The deep-sea supports the largest biosphere on Earth, regulating climate, and containing vast natural resources (Gage and Tyler, 1991; UNEP 2007). It represents a part of our planet that challenges conventional wisdom, hosting uniquely rich, complex and vulnerable ecosystems, such as cold-water corals, vents and seeps to cite a few. In the depths, both species and habitat discoveries remain high until today while current estimates of species richness range between 500 000 and 1.0 million species in the deep sea (Koslow, 2007). With these unexpectedly high estimates for species richness (Grassle and Maciolek, 1992; Levin et al., 2001), the deep sea is suggested to have one of the highest biodiversity of the planet (Ramirez-Llodra et al., 2010). This vast world of extreme physical conditions influences substantial adaptive radiations (i.e., the evolution of new species complexes) which lead to extraordinary

evolutionary novelty and basically contribute to biodiversity (Van Dover, 2000; Lorion et al., 2009). However our understanding of the mechanisms driving deep-sea life and ecosystems biodiversity are still uncertain (Gage and Tyler, 1991; Levin et al., 2001).

Sadly, as we discover and expand our knowledge on the extraordinary curiosities of the last frontier on Earth, we also realize and gather evidence for a strong footprint of human activities along the bathyal depths. Understanding the deep sea, its role in Earth's system more fully and how to regulate it for sustainable exploitation have been recognized as critical questions (UNEP, 2007; Danovaro et al., 2008). Beyond academic knowledge, this information may have important economic impact, as Europe relies on the marine environment for trade, food, resources, and jobs. In particular, Europe's deep-ocean margins form a relatively narrow zone, which extend from the Arctic to the Iberian margin along the Atlantic Ocean, and from the western Mediterranean Sea to the Black Sea, representing a total distance of 15,000 km. This deep-ocean frontier (i.e., the margin slope extending from the shallow shelf break at about 200m depths to waters of more than 4,000 m deep) covers 3 million km^2, which is about one third of the surface covered by Europe's landmass. Europe's continental margins are of critical importance as they lie within Europe's Exclusive Economic Zone (EEZ) and are therefore of direct interest for the exploitation of biological, energetic and mineral resources. Adopting the concept of the "Knowledge Economy" (i.e., an economy in which the generation and exploitation of knowledge play the predominant part in the creation of wealth, Drucker, 1969), the European commission is now supporting several consortia of collaborators attempting to tackle the many knowledge gaps on deep-sea ecosystems, with an emphasis along the margins. For instance, the HERMIONE project (Hotspot Ecosystem Research and Man's Impact On European seas, EC-FP7, from 2009 to 2012) focuses on deep-sea 'hotspot' ecosystems (i.e., supporting high species and specimens diversity) including submarine canyons, chemosynthetic environments, cold-water coral reefs, and seamounts, as well as the open slopes and deep basins. HERMIONE investigates the ecosystem's interconnection, dimensions, distribution and functioning, including the potential anthropogenic impacts (e.g., disturbance and climate changes). The eventual objective of HERMIONE is to provide a discussion platform between scientists, stakeholders and policy makers as well as with the public to support governance of the deep sea, sustainable management and conservation of these ecosystems. It includes 38 partners from 14 countries and has built strong links to intergovernmental organizations ranging from the UNEP (United

Nations Environment Programme) to nongovernmental bodies such as WWF (World Wildlife Fund), IUCN and to international initiatives such as the Census of Marine Life (CoMl). In addition, the project also strengthens some collaboration with private groups such as the hydrocarbons company Statoil Hydro, which took a significant share in the project.

The Dark Realm: Ecosystem, Habitats and Benthic Biodiversity

Photosynthesis is a process using the energy from sunlight to convert carbon dioxide into organic compounds, especially sugars, and releasing oxygen as a by-product. Since they can create their own food, photosynthetic organisms are called photoautotrophs (i.e., primary producers). Oceans, thanks to phytoplankton, are responsible for half of the total net primary production on Earth (Behrenfeld et al., 2006). They have a major role in carbon re-cycling, allowing the fixation of carbon from inorganic into organic matter and transferring it to the deeper layers by passive or active transport.

The lack of photosynthetically-usable sunlight (i.e., light penetrates the water column but is too little for photosynthesis) below the euphotic zone (or epipelagic) is a unique and shared characteristic of deep-sea ecosystems (Thistle, 2003). Known to occur from around 250m, it also coincides with the maximum depth of seasonal variability in temperature, the seasonal thermocline (Robinson et al., 2010). The pelagic deep ocean between 200 m and 1,000 m deep is called the mesopelagic or ‘twilight’ realm of the world’s oceans (Robinson et al., 2010) and coincides with the vertical gradient of temperature, known as the permanent thermocline. It is also where 90 % of the organic carbon annually produced by surface waters is respired back to carbon dioxide (Robinson et al., 2010). Buesseler and colleagues (2007) showed that rapid biological consumption and remineralization of carbon by resident biota within the ocean’s twilight zone can reduce the transfer efficiency of the downward particulate organic carbon (POC) flux, and therefore attenuate carbon dioxide sequestration in the deep sea. Below 1000 m depths in the immense bathypelagic zone, sunlight cannot penetrate anymore. At the benthic boundary layer near the sediment water interface, physical and biological interactions occur with the bottom, and the altered pelagic ecosystem is called benthopelagic (Ramirez-Llodra et al., 2010).

Most of the deep sea is hence heterotrophic and depends on food material sinking from the euphotic zone to the benthos in the form of small particles (Levin et al., 2001). Benthic deep-sea biota and communities are therefore adapted to life in environments with relatively low and/or sporadic levels of available energy and food (Gage and Tyler, 1991). Exceptions are only in the limited areas of reducing environments (chemosynthetic ecosystems) such as hydrothermal vents, and seep habitats where the primary production is made by chemoautotrophic bacteria using chemical sources rather than sunlight (Van Dover, 2000; Levin et al., 2001). However, regional and seasonal variations occurring in ocean's surface production result in spatio-temporal differences of organic matter input to the seafloor (Beaulieu and Smith, 1998). Recent studies also accumulated evidence of seasonal (e.g., internal tides, deposition of phytodetritus) and episodic disturbances (e.g., benthic storms) that alter the sea-floor communities in some areas (Gage, 2003). Abyssal plains, the largest body of Earth, were long regarded as monotonous and poor environments with no dynamical processes over short to long time-scales (See Ramirez-llodra et al., 2010). The abyssal sediments, consisting mainly of biological planktonic particles, so called oozes, can reach thousands of meters in thickness. Abiotic parameters, including temperature (about 2°C), salinity (around 35), dissolved oxygen (5–6 ml l^{-1}), pressure (which increases at 1 atmosphere every 10m depth), and absence of light were thought to be mostly uniform, with exceptions only in the Mediterranean and Black Sea and in Oxygen Minimum Zones (OMZs). However, episodic high-quality food resources were shown to affect abyssal communities (reviewed in Tyler, 1988), while some deep-sea trenches (> 6,000 m) were also characterized by considerable organic matter fluxes (Danovaro et al., 2003). Gage and Tyler (1991) suggested seasonal food input as an important factor structuring benthic biodiversity on a temporal scale and allowing the coexistence of species in the deep sea, while others suggested competition and disturbance as a source of temporal patchiness and influencing community succession (e.g., Grassle and Sanders, 1973).

Energy-rich habitats (e.g., hydrothermal vents, seeps and whale falls), although scattered (e.g., 10s of metres, isolated by 100s of kilometres) sustain truly extraordinary physiological adaptations leading to outstanding communities highly distinct from the remaining deep-sea environment (e.g., Van Dover, 2000; Smith and Baco, 2003). Yet, these very specific environmental niches and oases of life show much lower levels of diversity than in the nearby deep-sea floor. As mentioned earlier the deep-sea sediments are often

characterized by high values for local diversity estimates on the associated benthos. Several models are put forward to explain this high biodiversity including the intermediate disturbance hypothesis (IDH, maximum diversity at intermediate levels of disturbance frequencies or intensities, Connell, 1978) and the patch mosaic model (i.e., small-scale patches of food and disturbance create microhabitats in space and time, allowing specialized species to respond and coexistence of a large number of species; Grassle and Sanders, 1973). Although the observed diversity patterns might not be explained by a single factor, unique low levels of nutrients in the deep sea are suggested to exert remarkable selective constraints on organisms allowing amazing adaptations (Ramirez-Llodra et al., 2010). Food input (strong limitation, temporal deposition, type, quality, quantity), flow regime, bottom-water oxygen concentration, sediment heterogeneity and biotic disturbance might actually all together be important factors structuring benthic biodiversity in the deep sea (Levin et al., 2001).

In addition, the variability of habitats found in the deep sea (from microniches to regional scales, see Figure 2) is definitely a significant factor supporting one of the highest biodiversity levels on Earth (Grassle and Macioleck, 1992; Snelgrove and Smith, 2002). For instance, seamounts have a specific topography which creates different habitats characterized by particular hydrography, substrate type and productivity which influence great levels of biodiversity and distinct faunas. Also continental margins are among the most geologically diverse components of the deep-sea floor, exhibiting high habitat and ecosystem heterogeneity, including open slopes, other regions of complex topography and hydrography such as submarine canyons, cold-water corals and cold seeps and eruptions (Levin et al., 2010).

The sharp topographical variability of the seafloor together with environmental gradients of food supply, oxygen, and tectonic activity create a series of different habitats with unique characteristics (Levin et al., 2001). Especially, the margins host hotspots of biodiversity, such as cold-water coral ecosystems, which are suggested as the most remarkable features in term of biodiversity (Freiwald et al., 2004). In these ecosystems, biogenic structures such as scleractinian corals can be seen as ecosystem engineers. They influence flow and geochemical regimes, and provide a diversity of substrates for a highly diverse associated fauna. Numerous species consequently receive a stable surface for

attachment, have access to many food particle fluxes, and benefit from an aggregation site for mating.

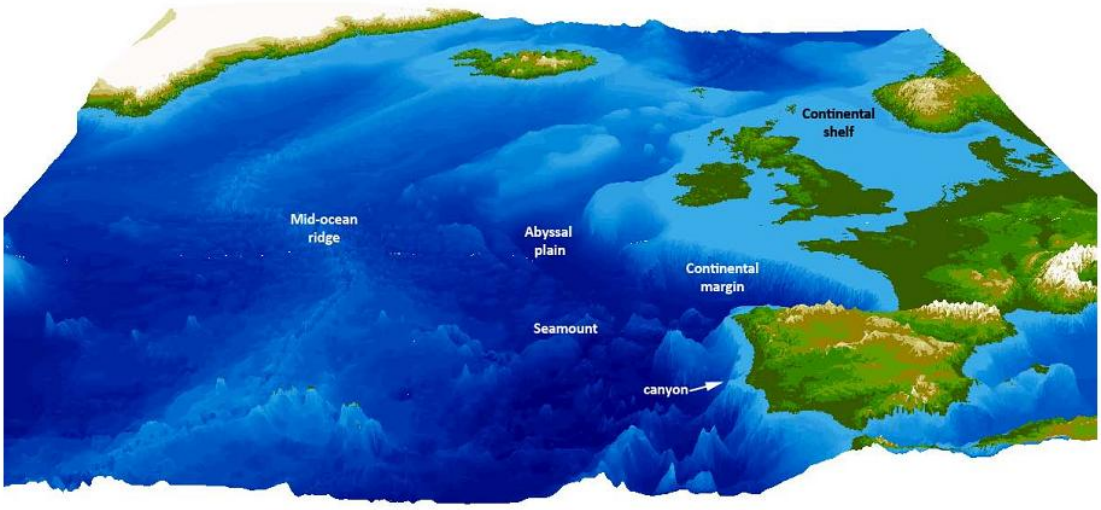


Figure 2**.** The NE Atlantic seafloor showing some of the distinct deep-sea ecosystems: continental margins – which can include canyons (arrow), cold seeps and cold water corals, abyssal plains, seamounts and the mid-ocean ridge, where hydrothermal vents are found. © Age Høines, MAR-ECO (an ocean exploration initiative within the global Census of Marine Life, CoML, From Ramirez-Llodra et al., 2010).

Cold-Water Coral (CWC) Ecosystems

Coral reefs, once believed to be restricted to warm shallow waters in the tropics, have been discovered far below the sunlit surface in dark, cold, nutrient-rich waters. These reefs are underwater oases, biological treasures, and habitats for many invertebrates and commercially important fish species. Found in all the world's oceans, almost from pole to pole, cold-water coral reefs may even rival in size and complexity those in warmer, shallower waters in tropical and subtropical regions. However, the reefs are slow growing, particularly vulnerable, and must urgently be preserved for future generations from further damage. These reefs are indeed at serious treat (e.g., within the reach of one of the most destructive human activities in deep waters, bottom trawling). Nevertheless, cold-water coral reefs and other high-seas habitats, beyond national jurisdiction, are starting to appear on political and governmental agendas and to influence international political decision making (Freiwald et al., 2004).

Fishermen in Scandinavian waters were the first to observe cold-water corals, when they accidentally encountered specimens in their nets. This chance discovery led to the first observations and drawings of cold-water coral species (Pontoppidan, 1753). In his account of the natural history of Norway, the Right Reverend Erich Pontoppidan, Bishop of Bergen and member of the Royal Academy of Sciences at Copenhagen indicated the coral being

absorbent, refrigirative, emollient, astringent and strengthening….. Coral beads worn round the neck were a preservative against the apoplexy, the plague and other contagions

So at that time, deep-sea corals were employed for 'medicinal' uses. The white coral species *Lophelia pertusa* was scientifically described by Linnaeus in 1758. From then on, the growth of deep-water fisheries in the late 18th to early 19th century as well as dredge samples from the Porcupine Seabight in the 19th century (Thomson, 1873) provided many more cold-water coral records. However, the records remain anecdotic. Coral reefs were mostly regarded as restricted to well-illuminated tropical seas, not as inhabitants of the dark and cold waters of higher latitudes.

It is only during the last decades that the development of acoustic survey techniques, access to submersibles and ROV's, and the mapping of large areas revealed the scale and abundance of cold-water coral ecosystems. Reports of single colonies of corals (e.g., in the Galicia Bank; Duineveld et al., 2004), large reef-like structures some kilometers in length (e.g., along the Sula Ridge of Norway; Freiwald et al., 2002) or the presence of small mounds covered with a thriving living cold-water coral fauna to giant carbonate mounds (e.g., Henriet et al., 1998, 2001; Huvenne et al., 2005; Foubert et al., 2005; Mienis et al., 2006; Kano et al., 2007; Foubert et al., 2008) along the European continental margin have challenged conventional views. A cluster of European research projects has focused on the enigmatic relationship between cold-water coral growth and the development of such mounds in the last decade (e.g., GEOMOUND, ECOMOUND, ACES, EURODOM) and teamed up to propose and achieve a major Ocean drilling action: the Integrated Ocean Drilling Program (IODP) Expedition Leg 307 in the Challenger Mound, Porcupine Seabight (see Foubert and Henriet, 2009). While old but extensive coral reports such as Joubin (1922a,b) or Le Danois (1948, who described the so-called ' massifs coralliens') were neglected for many decades, cold-water corals are now being rediscovered and currently reported on many continental margins and shelves, at seamounts, in fjords and in canyon systems, and found to be distributed in all world oceans (Northeast Atlantic Ocean: e.g., Wilson, 1979a; Jensen and Frederiksen, 1992;

De Mol et al., 2002; Fossa et al., 2005; Mienis et al., 2006; Reveillaud et al., 2008 this thesis; Roberts et al., 2009; Le Guilloux et al., 2009; Northwest Atlantic Ocean: e.g., Wareham and Edinger, 2007; Cordes et al., 2008; Davies et al., 2010; Indian Ocean: e.g., Rogers, 1999; the Pacific Ocean: e.g., Etnoyer and Morgan, 2005; Stone, 2006) and across the Mediterranean Sea up to the Black Sea (e.g., Zibrowius 1980; Taviani et al., 2005, See Figure 3).

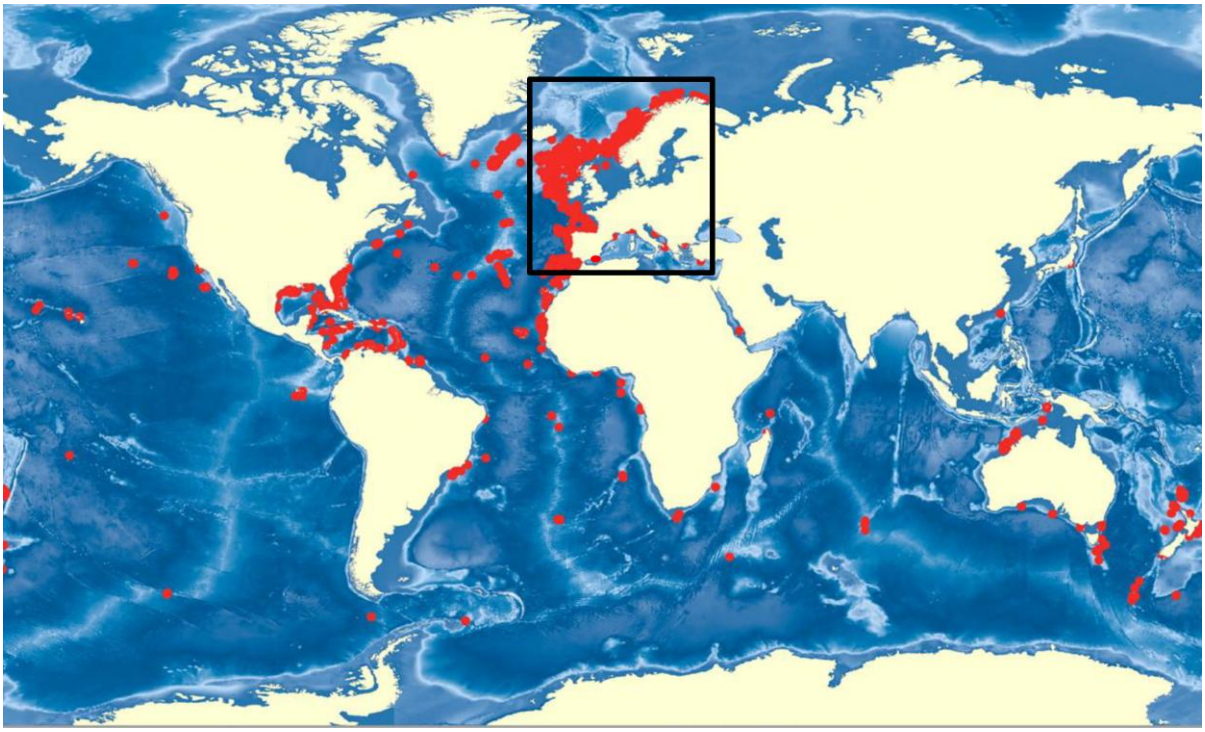


Figure 3. Global distribution of reef framework–forming cold-water corals. The black square indicates the study area of this dissertation (modified from Roberts et al., 2006).

Cold-water corals are azooxanthellate (i.e., lacking symbiotic algae) and are recurrently reported from sites with increased food supply and considerable water movement caused by currents or internal tidal waves (Jensen and Frederiksen, 1992; Frederiksen et al., 1992). Duineveld and colleagues (2004) showed from sediment trap analyses that important sources of POC (Particulte Organic Carbon; phytodetritus, fecal pellets, and zooplankton) were found to feed cold-water coral communities on the Galicia Bank in the NE Atlantic. Reefs and mounds were also observed to cluster in ‘‘provinces’’ where specific hydrodynamic and food supply conditions favor coral growth (Roberts et al., 2006). Cold-water corals are restricted to water temperatures between 4 and 13°C (Freiwald, 2003). They are found between 50 m and 4,000m depth in the Atlantic Ocean (Grasshoff, 1982) and ca. 6,300 m deep in the Pacific (Keller, 1976). Tropical coral reefs are built by numerous species

of corals while the reefs in the Atlantic are mainly built by the two scleractinian (i.e., stony corals) species *Lophelia pertusa* (Linnaeus 1758) and *Madrepora oculata* (Linnaeus 1758), sometimes with two or three other species of branching corals such as *Dendrophyllia cornigera* (Lamark, 1816) and *Solenosmilia variabilis* Duncan, 1873 (Jensen and Frederiksen, 1992) or *Stylaster gemmascens* (Van Soest et al., 2007a). In addition to the scleractinians, cold-water corals also include the order Octocorallia (soft corals), Antipatharia (black corals), and Stylasteridae (hydrocorals; Roberts et al., 2006). Living in thickets or in a reef, stony coral reefs have a three dimensional complex structure, providing ecological niches for many species (i.e., fishes and a multitude of invertebrates, such as bivalves, brachiopods, echinoderms as well as bryozoans; Jensen and Frederiksen, 1992; Freiwald et al., 2004). Recent research shed light on these extraordinary ecosystems, suggesting they have similar levels of biodiversity as shallow-water tropical reef communities (Jensen and Frederiksen, 1992). Yet, quantitative biodiversity studies are few and the functional relationships between species on cold-water coral reefs remain unknown. A better understanding of these 'hotspots of biodiversity' would require a more systematic approach in biodiversity studies (richness, endemism, spatial distributions, historical biogeographic affinities) of the benthic communities and an accurate assessment of the spatial genetic structure and population dynamics of the corals and associated species.

Both live and dead corals have been found to participate in the development of coral reefs (Wilson 1979b). The outer parts of the coral bank support the living coral tissue and polyps, while the inner part comprises dead and decaying coral mixed with sediments (Wilson 1979b). As a coral develops, polyps in the terminal branches of *Lophelia* die, and the inner parts of skeleton are being degraded by boring organisms (notably, clionid sponges). The bioeroded skeletons become increasingly vulnerable to mechanical breakage (Wilson 1979b), and dead branches falling on the seabed extend the perimeter of the reef patch. These processes create the reef framework that over time initiates localized mound formation (Huvenne et al., 2005; Roberts et al., 2006). The associated fauna is predicted to change with the stage of reef growth and available microhabitats (Roberts et al., 2006). Mainly dead corals were assumed to provide a substrate for a rich associated fauna (Jensen and Frederiksen, 1992).

Cold water coral reefs actually occur in mid-depth (500 to 1,200 m depth) water masses below the Eastern North Atlantic Water (ENAW). As far as to the Porcupine Seabight reefs are found within the upper depth limit of northward moving Mediterranean Outflow Water (MOW). More to the north, corals occur along the Norwegian Margin in North Atlantic Current (NAC) water, below the low saline surface currents of the Norwegian Coastal Current (NCC; Figure 4).

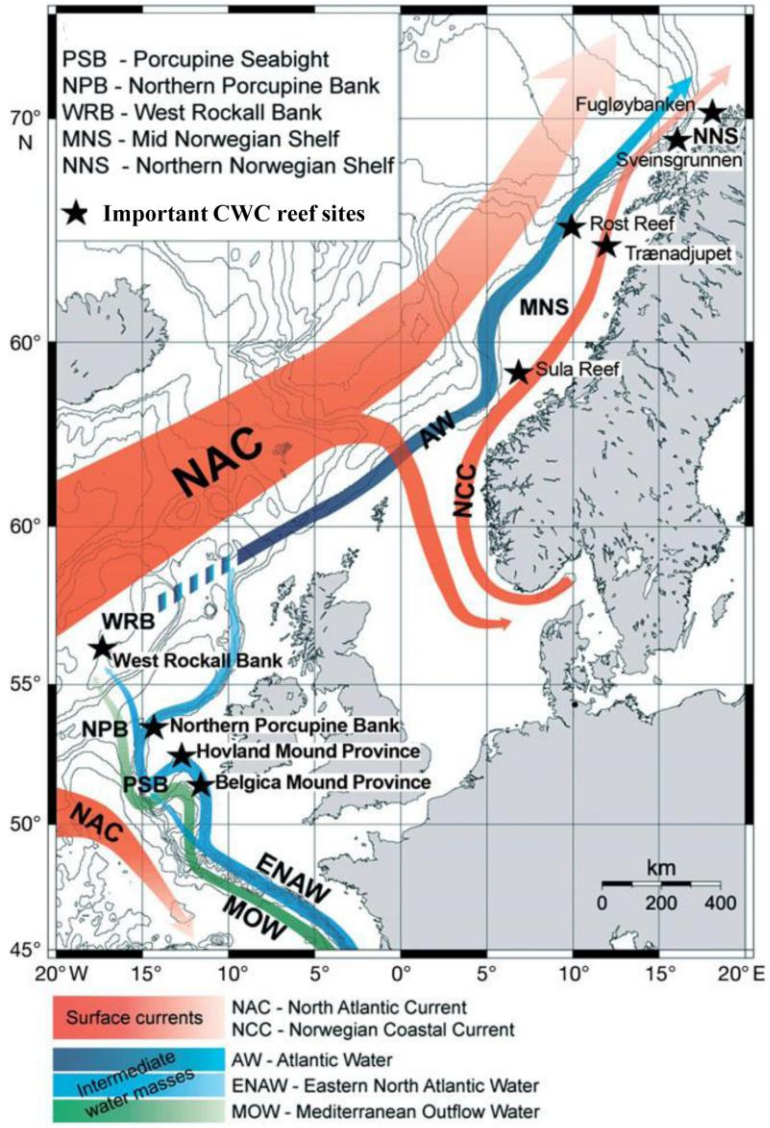


Figure 4: Locations of cold-water reefs along the Celtic and Norwegian Margin and prevailing current regimes. Red arrows indicate surface currents of the North Atlantic Current (NAC) and the Norwegian Coastal Current (NCC), blue and green arrows indicate intermediate water mass circulation of Mediterranean Outflow Water (MOW), the overlying Eastern North Atlantic Water (ENAW) and Atlantic Water (AW; Modified from Dullo et al., 2008).

Paleoecological U/Th and ^{14}C datings suggest the occurrence of constant cold-water coral growth over the last 50,000 years in southern refugia (e.g., the Mediterranean Sea, the region off NW Africa, and the mid-Atlantic ridge beyond the southern limit of the ice sheets; Schröder-Ritzrau et al., 2005; Roberts et al., 2006, Wienberg et al., 2009). However it is also suggested that during interglacial periods, corals may have been driven by post-glacial currents from these putative speciation centers towards northern latitudes, such as Scandinavia. There, reefs are shown to date from the Holocene after the retreat of the Pleistocene ice sheet (10,000 to 14,000 years ago; Schröder-Ritzrau et al., 2005; Roberts et al., 2006). By their fragmented, island-like distribution, questions about CWC reefs connectivity and the exchange of individuals among these geographically separated populations are important, as it remains a critical issue to know if the coral and associated fauna are sustained by larval replenishment or whether they are isolated (Van Soest et al., 2007). Recent genetic studies on *Lophelia pertusa* showed that the coral species could not be considered as one panmictic population in the North East Atlantic, but rather formed distinct offshore and Scandinavian fjord populations (Le Goff-Vitry et al., 2004, Le Goff-Vitry and Rogers, 2005). On the other hand, the same authors showed a moderate gene flow along the continental slope. The genetic analyses revealed highly variable levels of genetic diversity, inbreeding, and asexual reproduction from site to site (Le Goff-Vitry et al., 2004). These results are important to support sound conservation measures for these rich but vulnerable deep-sea resources.

Indeed, with the depletion of shallow-water fish stocks and the improvement of technologies, the commercial exploitation of the deep-sea environment like CWC reefs is occurring at increasing intensities and poses a serious threat to these complex but really fragile ecosystems. We are gathering widespread evidence that anthropogenic impacts threaten cold-water coral ecosystems in three ways: (i) bottom trawling for deep-water fish causes severe physical seabed damage (ii) hydrocarbon exploitation through drilling and mining activities, although much more localized, have potential impacts on reef fauna and on local extinction, and (iii) ocean acidification, through the significant and sharp increase of atmospheric carbon dioxide, has potentially insidious effects on coral calcification (Roberts et al., 2006).

Sponge Biodiversity Studies in Cold-Water Coral Ecosystems

Sponges constitute a remarkable component of cold-water coral reef fauna in the North Atlantic, and appear as one of the most species-rich groups (e.g., on the Faroe shelf coral banks, Jensen and Frederiksen, 1992; Rogers, 1999). The three Poriferan classes (Hexactinellida, Demospongiae and Calcarea) are represented in cold-water coral ecosystems. Although a considerable body of literature focuses on sponge diversity in tropical shallow-water coral reefs, providing estimates of local reef and regional diversity (Alcolado, 1994; Diaz and Rützler, 2001*;* Hooper and Kennedy, 2002; Cleary et al., 2005; Becking et al., 2006; De Voogd et al., 2006, Worheide, 2006; Bentlage and Worheide, 2007; Worheide et al, 2008), most studies in cold-water corals employed an integral community approach with little attention for individual components other than corals (Jensen and Frederiksen, 1992; Bruntse and Tendal, 2001; Mortensen and Fossa, 2006). These studies focused on well-known sponge taxa and failed to observe or ignored lesser-known taxa. Generally, sponge biodiversity studies relied on trawling and dredging, therefore remaining qualitative (at most semi-quantitative; Barthel and Tendal 1993; Boury-Esnault et al., 1994). Taxonomists initially focused on large-sized, bright-coloured or prominent Porifera species and reported the concentrated occurrence of megabenthic sponge species *Pheronema carpenteri*, *Geodia* spp. and *Asconema aff. setubalense* associated with bathyal reefs (Rice et al., 1990; Klitgaard and Tendal 2001, 2004). However the extensive presence of small sized and morphologically cryptic sponges in cold-water coral reefs was widely noted (Stephens, 1915, 1921; Vacelet, 1969; Rogers, 1999). Biodiversity studies addressing the substantial diversity of deep-sea sponges associated with CWC reefs are limited to four recent papers: Longo and colleagues (2005) reported 39 species on a Mediterranean CWC reef (Santa Maria di Leuca); Van Soest and Lavaleye (2005) recorded 95 species on an Irish CWC reef (Rockall Bank); and later on Van Soest and colleagues (2007a) revisiting this area as well as a reef area on the northwestern Porcupine Bank reported 191 species; finally, Roberts et al., (2009) listed 100 sponge species from the Mingulay reef complex in the Sea of the Hebrides west of Scotland. These diversity studies confirmed that species forming thin encrustations were by far the most common growth form among CWC reef sponges in the NE Atlantic (Van Soest and Lavaleye, 2005).

The nature of the relationship between corals and sponge diversity (richness and evenness) remains complex and is apparently influenced by several parameters, including the state of corals (live or dead) and the extent of coral cover. The presence of coral represents a primary condition for sponge presence and abundance, as exposed sediment lacking corals in the surrounding reef environment (i.e., muddy bathyal bottoms) were found with very poor sponge assemblages in the Mediterranean Sea and in Ireland (Longo et al., 2005; Van Soest et al., 2007a; personal observation). On the other hand, no species were found exclusively on *Lophelia pertusa* and most species associated with the corals are present on other hard substrata in the local geographic area (Jensen and Frederiksen, 1992). Van Soest et al. (2007a) found that the sediments including 'dropstones' surrounding the reefs formed an impoverished 'reef' habitat for sponges instead of a separated one. Bathyal sponge reef faunas are then characterized by the lack of any dominant sponge species and by a high heterogeneity over large geographic areas (e.g., eveness J' of 0.81-0.97 in the Rockall Bank, Van Soest and Lavaleye, 2005). In 2005, Van Soest and Lavaleye compared the 95 species they reported in bathyal coral reefs of Rockall Bank to ancient sponge collections. These included the list of Carter (1874; 72 sponge species -excluding Calcarea- sampled at 520-1550 m, west of the British Isles and Ireland) and the one of Stephens (1915, 1921; 91 sponge species caught at 396-1350 m, on *Lophelia* beds out on the continental slope of West Ireland, Porcupine Bank area). The authors found very few shared sponge species (19 in Carters' record and 31 in Stephens list) between these adjacent regions and confirmed that typical local deep-sea sponge communities appeared to be present at small scales, including in *Lophelia* reefs (Klitgaard and Tendal, 2001; Van Soest and Lavaleye, 2005).

It is yet noteworthy that numerous species inhabiting CWC reefs are encrusting sponges on dead coral fragments. The latter are also often found excavated close to the living coral tissue, and filled with sponge tissue of boring species such as *Aka infesta* or *Alectona millari* (Jensen and Frederiksen, 1992; personal observation), sometimes together with the common species *Pione vastifica* (Jensen and Frederiksen, 1992) and *Entobia* spp. Sponge community analysis, however, showed **no correlation between species richness and dead coral cover.** For instance, a particular dead coral branch showed only three individuals belonging to a single species (e.g., *Hexadella dedritifera*), while others have been seen to contain up to 22 sponge individuals belonging to 15 different species (Rockall bank, Van Soest and Lavaleye, 2005). In addition, the surface of dead corals was not entirely covered

with sponges or other invertebrates (Van Soest and Lavaleye, 2005), suggesting a possible lack of competition. Depth, like in shallow-water tropical reefs (e.g., De Voogd et al., 2006), was shown to structure sponge assemblages in CWC reefs in the Mediterranean Sea and in the NE Atlantic (Longo et al., 2005; Van Soest and Lavaleye, 2005). In addition, the **presence of live corals was identified as one of the most important structuring factors in sponge community composition and diversity within reefs**. Recent statistical analyses performed on sponge communities (Van Soest and Lavaleye 2005, Van Soest et al., 2007a) highlighted three key points: (i) **species richness was higher in the presence of live corals** than in the absence of live corals (presence = 1; absence=0) ; (ii) however surprisingly species richness was negatively correlated with the percentage of live coral cover so that **the largest number of sponge species was found associated with relatively low live coral cover**; (iii) some species were strongly associated with live coral cover (e.g., *Hexadella dedritifera*) while the occurrence of other species was found negatively related to the presence of live corals (e.g., *Rossella nodastrella*).

Therefore, although many sponge species inhabiting CWC reefs encrust mainly on dead coral fragments, they also profit from the same abiotic conditions that favor coral growth (i.e., increased food supply) and they share their preferred habitat with corals. Although we have no evidence for competition between sponge and corals, the negative correlation between the species *Rossella nodastrella* occurring massively in Irish cold-water coral reefs and the presence of live coral cover (Van Soest et al., 2007b) may possibly be the result of competitive exclusion. In contrast, the species *H. dedritifera*, found to encrust dead coral branches was seen to overgrow other sponge species (personal observation and see CHAPTER III, this thesis).

Sponge Putative Functions and Bioprospection in the Deep Sea

Long disregarded, deep-water sponges can also form deep-sea sponge grounds (i.e., aggregations of large sponges that form structural habitat), which may play a similar role to that of CWC reefs in the deep sea. The wider availability of deep-sea survey, sampling and mapping have allowed the recent discovery of giant glass (Hexactinellid) sponge reefs off the coast of British Columbia, western Canada (Hogg et al., 2010). Siliceous sponge reefs were most widespread during the Late Jurassic, forming a huge sponge reef belt more than 7,000

km in length on the northern shelf of the Tethys Sea and the North Atlantic basins. Although they were formerly thought to be extinct, their modern finding permitted a better understanding of fossil taxa and the process of sponge reef building. Sponges belonging to Demospongiae or Hexactinellid (i.e., spicules of silica SiO_2) were also found to occur in high concentrations in Antarctic waters, also called the 'sponge kingdom'. When hexactinellid sponges die, their skeletal disintegrates and form spicule mats, which increase biomass of benthic species (Bett and Rice 1992). Therefore, disturbance and removal of spicule mats may cause damage to the present distribution of sponges, and prevent future recruitment and recovery of the structural sponge habitat. These diverse deep-sea sponge grounds are characterized by a slow-growing process and the high biomass and three-dimensional structure of sponges support of a high biodiversity. However, these structures are highly vulnerable to human impact such as trawling, documented during the past decade (Hogg et al., 2010).

Sponge reproduce both sexually and asexually (by means of fragmentation, budding and gemmulation), although clonality seems to have a structural role only at small spatial scales (i.e., a few meters, Calderon et al., 2007). They can be gonochoristic or hermaphroditic, viviparous and brooders or oviparous and spawners (Maldonado and Riesgo, 2008), but most of them have an indirect development, releasing lecitotrophic larvae with short planktonic life cycles, reduced dispersal capabilities and phylopatric behavior (Maldonado and Bergquist, 2002; Maldonado, 2006). Consequently, sponges are thought to have a short dispersal phase and high population structure (Duran et al., 2004; Calderon et al., 2007). However, this remains to be further investigated in the deep sea.

Maldonado and Uriz (1999) actually drew attention to the strategic role of sponge reproduction in spatially separated populations, where species occur in discrete populations. In marine fragmented areas, such as the deep-sea, currents may play an important role (for both asexual and sexual reproduction, see Figure 4). It is indeed possible that sponge fragments (clones) are transported by ocean currents to settle and colonize new areas.

The majority of sponges associated with cold-water coral ecosystem can be assigned to four different growth forms (Figure 5):

1- Thin, small hairy crusts, mainly belonging to the viviparous order Poecilosclerida (e.g, genera *Hymedesmia*, *Plocamionida)* are presumably short-lived, surviving long enough to reproduce (Van Soest and Lavaleye, 2005). Their body covered with hairy spicule assemblages suggests a defensive adaptation.
2- Thin, soft crust, such as the abundant *Hexadella* sp Topsent, 1896. The absence of skeleton in such 'keratose' genera (i.e., taxa which lack spicules) might be compensated by important chemical defense mechanisms.
3- Thin skin over an essentially 'empty' body (Hollow-bladder-like forms, also generally Poecilosclerida, (e.g, genus *Desmacella* which may trap food via the mucus in their body).
4- Massive, large, highly silicified forms, (e.g., genera *Asconema*, *Geodia*) appear as long-lived specimens, which invest a lot of energy in the silica body and have therefore a low biomass.

These different morphological forms may imply distinct evolutionary strategies to handle the deep-sea environment regimes of low input of organic matter over long periods of time. But they could also imply a variety of different functions in CWC reefs.

Sponges are filter feeders, except for a small family of carnivorous species. They are capable of filtering thousands of liters of water per day. The sponge body is organized around a system of canals which conducts the water current between inhalant (pores or ostia) and exhalant apertures (oscules). **Sponges have no true tissues or organs** but utilize independent cellular activity to carry out all vital body functions (e.g., feeding, reproduction, secretion, etc..). **Feeding functions** involve an epithelial layer of pinacocytes (i.e., which isolate the organism from the environment) and a pumping choanocyte (collar cell) layer, both only a single cell deep. Enclosed between these two layers is the mesohyl, a region which corresponds to the connective tissues of higher organisms and which is involved in all other functions (e.g., digestion, spicule secretion).

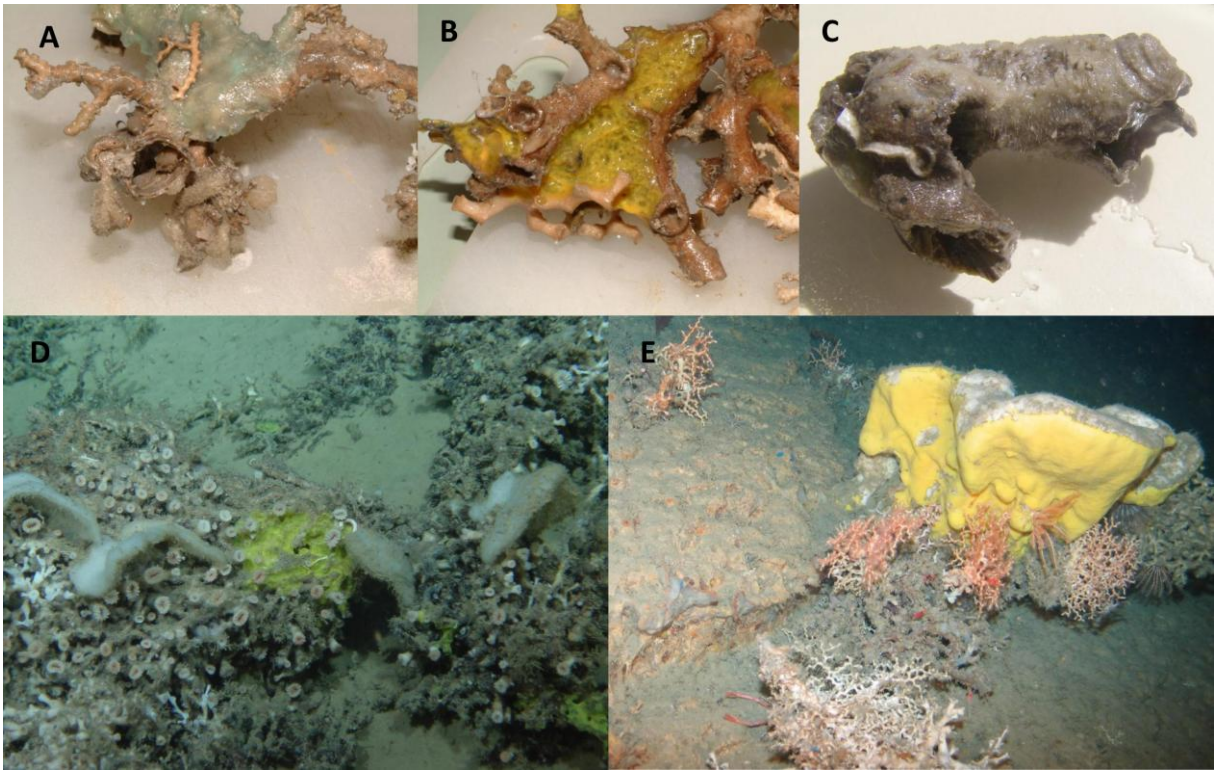


Figure 5. Sponges associated with cold-water coral ecosystems. (A) *Desmacella inornata*. (B) *Hexadella dedritifera*. (Images courtesy of R.Van Soest, Zoological Museum of the University of Amsterdam, The Netherlands. (C) *Plocamionida ambigua.* (D) *Poecillastra compressa* and *Hexadella sp* from CWC reefs in Santa maria Di leuca, Mediterranean Sea (Copyright Institut Francais de Recherche pour l'Exploitation de la Mer, IFREMER). (E) Massive demosponge covered by a thin encrusting species, probably *Hexadella sp*, in the Bay of Biscay, France (Image courtesy of L. De Mol, Renard Centre of Marine Geology, Belgium, See De Mol et al., 2010).

In shallow-water coral reef systems, the diverse and abundant sponge community is thought to play an important functional role in the ecosystems by turning over the water column within the reef (Diaz and Rützler 2001; Scheffers et al., 2004). Sponges of shallow-water reefs were also found to have important functional roles in reef bioerosion and reef creation, stabilization, and consolidation; it was shown that sponge can mediate the consolidation of corral rubble by seeding them until carbonate secreting organism growth. Sponges are then used as microhabitat by a large number of small invertebrates (e.g., polychetes, mollusks) and provide specific predation protection (e.g., by their presence on bivalve they reduce mortality from starfish perdation or they provide camouflage to crabs) while they are consumed by a range of predators (e.g., fish, crustaceans, mollusks). As such, sponge fulfil a number of important roles which may have major implications for ecosystem functioning (reviewed in Bell, 2008). It is highly likely that all these important ecosystem

functions apply to sponges in bathyal coral reefs. Sponges in temperate deep water reefs in the Mediterranean were shown to play important ecological roles in bioerosion (Beuck et al., 2007) whereas sponge feeding was found to be important to bentho-pelagic coupling in the deep sea (Pile and Young, 2006). In addition, Van Duyl et al., (2008) showed the assimilation of dissolved organic and inorganic carbon by the sponge-microbe consortia (e.g., sponge associated chemoautotrophic nitrifying prokaryotes) of the sponge species *Higginsia thielei* and *Rossella nodastrella* in NE Atlantic CWC reefs. Actually, more than 40 % of the biomass of sponges may consist of microorganisms (including Bacteria, Archaea and Fungi; Vacelet 1975, Taylor et al., 2007). It is likely that these symbiotic associations provide the sponges, along with their filter-feeding capabilities, a suite of nutritional capabilities in their oligotrophic CWC reef environments. These highly diverse communities of sponge-associated Bacteria and Archaea (Taylor et al., 2007) may also open up new pathways in the ocean carbon cycle.

Moreover, the decomposition of bacteria-rich sponge tissue may result in the formation of a compact micropeloidal fabric (i.e., microbially-induced calcium carbonate precipitation) which can be observed in old sponge reef carbonates (Flügel, 2004). The calcification of these microbial aggregates is formed by the combination of (i) the attraction of calcium ions by the negatively charged microbial cell surface, (ii) the secretion of bicarbonate as final metabolic by-product by microbes (heterotrophes), and (iii) the presence of microbial consortia embedded in extracellular polymeric substance (EPS, secreted by the microorganims; Sanders et al., 2006).

Sponges have developed a wide range of highly effective chemicals against predators, spatial competitors, or as antifouling (e.g., Becerro et al., 2003). However in some cases it is not yet clear whether these compounds originate from the sponge itself, or from their symbiotic association with microbes. These compounds have a variety of activities (e.g., antibacterial, antifungal, anticoagulant, anti-inflammatory, antimalarial, antiviral, and anticarcinogenic) and have placed Porifera among the most prolific and promising producers of important compounds for the pharmaceutical industry (Faulkner, 2000). The pioneering work of Bergman and collaborators on sponge arabinose nucleosides, which were shown to

have bioactive properties, initiated an extensive search for potential marine drugs from sponges and other marine invertebrates (see Erpenbeck and Van Soest, 2007). For instance, verongid sponges produce complex brominated, tyrosine derived compounds, which show a wide range of biological activities (e.g., anti-HIV, but see Matsunaga et al., 2005) and several other groups of sponges are now of particular interest for the pharmaceutical industries.

However, a crucial step for sponge bioprospection is unambiguous species delineation (Wu et al., 1986; Morris and Andersen, 1989; Erwin and Thacker, 2007; Erpenbeck and Van Soest, 2007). This may be of particular importance in the deep sea, where so many species 'new to science', from all phyla, are recurrently reported. Approximately 8300 sponge species are currently recognized (WPD, Van Soest et al., 2011) but the true diversity of the phylum is expected to be twice as high, based on the available data supporting a high number of cryptic species in the Phylum Porifera (see Ref herein).

Morphology and Cryptic Species in the Phylum Porifera

Sponges represent a taxonomic group in which species identification is particularly difficult. Morphology of spicules and skeletal arrangement are traditionally the diagnostic characters to identify sponge species (Hooper and Van Soest, 2002). External characters such as texture, form, and coloration are frequently influenced by environmental factors (Palumbi, 1984; Jones, 1984; Schönberg and Barthel, 1997; Carballo et al., 2006). Conversely, spicule size and micromorphology are thought to be influenced by the environment under exceptional environmental conditions only (e.g., in particularly high or low silica levels; Maldonado et al., 1999). Nevertheless, the evolution of skeletal traits (including size, shape, arrangement and combination) is not fully understood, leading to homoplasies and erroneous classification. For instance, the genus *Plocamionida* (Porifera, Poecilosclerida) has excellent morphological markers (i.e., large styles, tornotes, acanthostrongyles, chelae). However, *Plocamionida* remains a group of sponges that are notoriously difficult to identify because the intra- and interspecific character variation is not well understood, and has given rise to disagreements between taxonomic experts.

Despite the extraordinary efforts to revise the classification system in Systema Porifera (Hooper and Van Soest, 2002), taxonomic and systematic uncertainty may therefore prevail. This especially applies to species with a low number of informative characters (Klautau et al., 1999; Knowlton, 2000) such as 'keratose sponges', which lack a mineral skeleton and

comprise taxa of the orders Verongida, Dictyoceratida and Dendroceratida. For example, the four verongid families are distinguished almost exclusively by the structure and composition of their spongin fibers (Bergquist and Cook, 2002). The quest for diagnostic morphological characters for unambiguous species identification in this group is hence a special challenge. The value of cytological features (i.e., the anatomy and cell composition) for sponge systematics has been investigated for a few sponges without skeleteon (e.g., Boury-Esnault et al., 1995), and while effectively resolving a few taxa, cytological studies remain scarce. Some species, such as *Hexadella* species (Porifera, Verongida, Ianthellidae) are fiberless and species delimitation is virtually impossible (Berguist and Cook, 2002). These features make many Porifera prone to cryptic species and as species boundaries may be confounded, this leads to an underestimation of the phylum's biodiversity.

The structural complexity of the **biochemical compounds** released by sponges has been suggested as an alternative to morphological characters (Van Soest and Braekman, 1999). However, Erpenbeck and Van Soest (2007) reviewed many pitfalls of sponge chemotaxonomy (e.g., issues related to the homology and homoplasy of characters) and concluded that biochemical compounds might be a source of additional characters when plotted against a robust and comprehsensive phylogenetic tree. The use of **DNA sequence** comparison in sponge systematics, which started in 1991 by Kelly-Borges and colleagues, provided vast amount of characters exceeding noticeably the resolution power of sponge compounds (Erpenbeck and Van Soest, 2007). Molecular systematics became an established field because evaluation of character homology appeared easier in DNA alignment (especially with protein-coding genes) than with morphological or biochemical characters. Also, molecular phylogenetics often uses a recognized model of evolution (i.e., nucleotide substitution models in Maximum Likelihood and Bayesian phylogenetic reconstructions). Molecular markers (via enzyme electrophoresis or DNA sequences) repeatedly revealed cryptic species and proved valuable in delineating species boundaries in Porifera (see review in Xavier et al., 2010a). Furthermore, the use of a comprehensive 128 gene data set allowed a recent phylogenomic study to confirm that the sponges are monophyletic (Philippe et al., 2009) and not paraphyletic as repeatedly suggested (e.g., Borchiellini et al., 2001). The resulting phylogeny had significant implications for understanding the origin of animals and the important changes that shaped the body plans of the early diverging metazoan (Fig. 6).

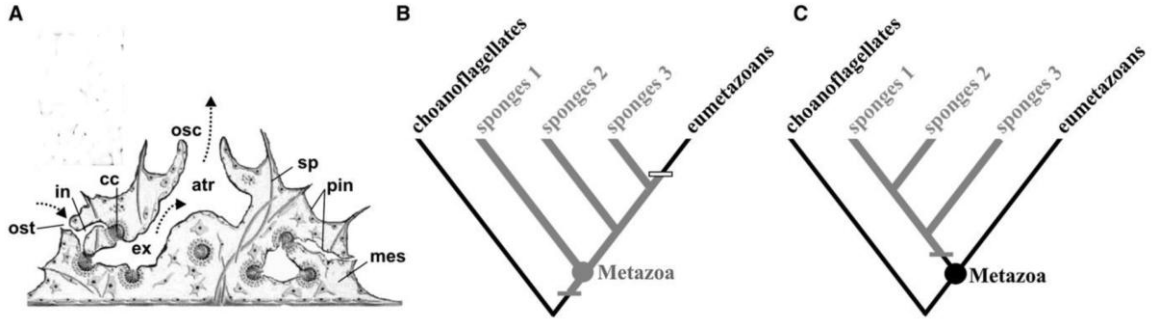


Figure 6. Characters of the Sponge Body Plan and Their Evolution. (A) Schematic section of an adult sponge (bottom). The arrows indicate the direction of circulation of water in the aquiferous system of the sponge. Abbreviations: atr, atrial cavity; cc, choanocyte chamber; ex, exhalant canal; in, inhalant canal; mes, mesohyl; osc, osculum (or exhalant orifice); ost, ostium (or inhalant orifice); pin, pinacoderm (thin epithelial layer, limiting the sponge body on its external surface and within the canals); sp, spicule. (B) Most parsimonious scenario for the evolution of sponge body plan characters, imposed on a scheme of sponge paraphyly. (C) Most parsimonious scenario assuming sponge monophyly. In (B) and (C), the gray branches indicate the presence of sponge body plan characters (aquiferous system, internalized choanocyte chambers, pinacoderm) and the black branches indicate the absence of these characters. The gray horizontal line indicates character acquisition; the hollow horizontal line indicates character loss. ‘‘Sponges 1, 2, and 3’’ correspond to the major lineages (silicisponges, homoscleromorphs, and calcisponges), of which exact branching order varies among published studies recovering sponge paraphyly (modified from Philippe et al., 2009).

Molecular Markers in Sponge Evolutionary Studies

In most phyla, the rapidely evolving mtDNA is the preferred marker to investigate species boundaries, to infer the phylogenetic origin of different lineages of organism as well as to afford phylogeographic studies (Avise, 2000). In addition, the use of the cytochrome *c* oxidase subunit 1 (COI), amplified with the universal primers of Folmer and collaborators (1994) was promoted in the ‘DNA barcoding’ life initiative (i.e., the DNA-based identification of species; see Hebert et al., 2003). Mitochondrial genes show an unexpected high substitution rate in mtDNA in mammals, especially at the third codon positions of protein-coding genes, compared to genes of the nuclear genome (Brown et al., 1979). Arguments to explain this high evolutionary rate for mtDNA included the effects of a high concentration of reactive oxygen species combined with an inefficiency of systems to repair DNA damage in mitochondria (Brown et al., 1979) and the fact that mitochondrial DNA was not covered by evolutionary conserved histone proteins, which might constrain rates of nuclear DNA evolution (Richter, 1995). Mitochondrial genes are generally maternally inherited (for an exception see Zouros et al., 1994) and nonrecombining (but for an exception

see Hoarau et al., 2002). Therefore, they are hierarchically branched and non-anastomosing even within sexually reproducing species and matrilineal genealogy can be reconstructed. MtDNA markers also have shorter coalescence times than their nuclear counterparts; they are expected to undergo lineage sorting three times faster than nuclear markers due to the fourfold smaller effective population size compared to nuclear DNA (Birky et al., 1983). However, a general high level of **mtDNA conservation was reported in diploblast phyla** (Shearer et al., 2002), and some difficulties in resolving taxonomic and phylogeographic relationships in sponges were reported (Duran et al., 2004; Wörheide, 2006). This appeared in sharp contrast to other marine Bilateria (e.g., bivalves, copepods; Shearer et al., 2002).

On the other hand, genetic studies above and below the species level have been performed using the COI partition (Erpenbeck et al., 2002; 2006a,b; Duran and Rutzler, 2006; Wulff, 2006; Blanquer and Uriz, 2007; Reveillaud et al., 2010, this thesis). In addition, the COI downstream I3M11 partition showed more resolution than the standard M1M6 partition (Erpenbeck et al., 2006b) and proved even useful to determine the genetic population structure of Caribbean and European sponges species (Lopez-Legentil and Pawlik, 2009; Xavier et al., 2010b).

The genes of the nuclear ribosomal DNA (rDNA), coding for the RNA component of the ribosome, have been used extensively in evolutionary studies of sponge species. The rDNA is a multigene family with nuclear copies in eukaryotes, organized in arrays of units that are arranged in tandem repeats. A single unit consists of the rDNA genes for 18S (small subunit), 5.8S, and 28S (large subunit) rDNA molecules which are separated by internal transcribed spacers (ITS-1 and ITS-2). The external transcribed spacer (ETS) and the intergenic spacer (IGS) separate the large and small subunit rDNAs. As a result of different forces acting across single repeat units, the different rDNA regions show varying degrees of sequence conservation, and therefore can be used for particular phylogenetic questions at different taxonomic levels (Hillis and Dixon, 1991). The small and large subunits are highly conserved and have been used for high level sponge phylogeny (18S: e.g., Borchiellini et al., 2004; 28S: e.g., McCormack and Kelly, 2002; Borchiellini et al., 2004; Erpenbeck et al., 2004), but in some cases for inter-specific studies (18S: e.g., Blanquer and Uriz, 2007; 28S: e.g., Blanquer and Uriz, 2007; Worheide et al., 2008). The rDNA 5.8S gene is too short to provide a robust phylogenetic signal. The faster evolving ITS region, however, has been

employed for population genetic studies and in sponge phylogeography (e.g., Wörheide et al., 2002). Nevertheless, the occurrence of potentially paralogous ITS copies can be problematic and can confound phylogenetic inferences (e.g., Wörheide et al., 2004). Variation among the multiple ITS copies is normally homogenized by a process called 'concerted evolution' (Elder et al., 1995), but this can be slow, and intragenomic polymorphisms can occur (Wörheide et al., 2004).

Highly variable nuclear intron sequences were suggested as novel markers for comparisons across populations and taxa (Hare, 2001). Degenerated exon- primed intron-crossing (EPIC) primers (i.e., located in conserved coding regions and flanking introns) readily available in the literature (e.g., Jarman et al., 2002), allowed the amplification of the second intron of the *ATP synthetase beta subunit* gene (*ATPSb*-iII), which was used successfully in sponge evolutionary studies (Bentlage and Worheide, 2007; Worheide et al., 2008).

Microsatellite markers were also developed in this group, showing to be really useful in fine scale studies on population structure and to unravel processes such as asexual reproduction (e.g., Blanquer et al., 2009) but their technical development seemed laborious and impeded in particular by high microbial DNA content.

Thesis Aims and Outlines

The overall aims of this study are to investigate the phylogenetic relationships of *small encrusting sponge* species in the Mediterranean Sea and along the North East Atlantic margin, from shallow-water to deep-sea coral ecosystems (Figure 7).

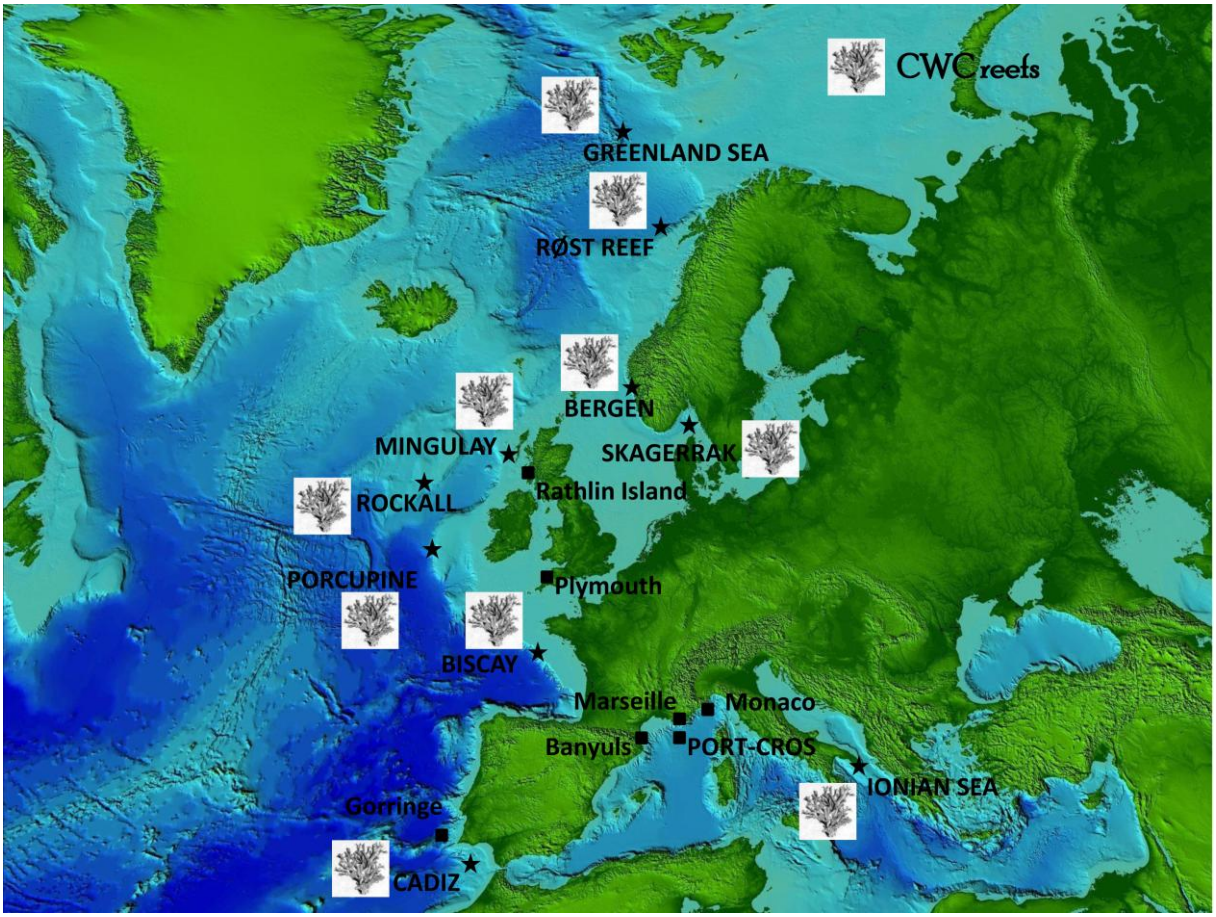


Figure 7. Map showing study sites of cold-water corals ecosystems (star shape) and other habitats (square shape). Map was provided by the project Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE). Sampling location are given in uppercase letters for deep-water samples (>50m) and in lowercase letters for shallow-water samples.

At the base of any biodiversity study there has to be a characterization of the habitat. My first aim was hence to compile literature on cold-water coral habitats and their distribution along the European continental margin. While a wealth of integrated studies focused on the cold-water corals in the Mediteranean Sea, the Gulf of Cadiz, the Irish to the Norwegian margins, studies on the distribution of scleractinian corals in the Bay of Biscay, although relatively numerous during the nineteenth and the first half of the twentieth century, were found to be scattered, sometimes unpublished. Knowledge about the current coral occurrence of the Bay of Biscay was actually limited. We therefore assembled published and non-published information on the occurrence of scleractinian corals in the Bay of Biscay (historical records from as early as 1830 up to 1995 and more recently collected information from two deep-sea cruises). A database of 347 records including 34 described species of scleractinians nicely documented that the slope of the Bay of Biscay is an important habitat

for cold-water coral reefs along the European margin (CHAPTER II). This study is published in Facies (2008, 54:317-331).

This cold-water coral literature compilation also allowed a general assessment of the richness, composition and distributional range of the deep-sea coral associated fauna, especially of the dominating deep-sea sponges. Historical datasets (Topsent, 1928; Stephens, 1915, 1921) and newly sampled material from Museum collections highlighted small encrusting sponges as numerous and with a broad distributional range. Although challenging to identify, we expected that their ecological and biochemical importance was likely to compete with conspicuous and megabenthic specimens. Some species like *Hexadella dedritifera* Topsent, 1913 showed a wide geographical distribution in the deep sea, over a distance of more than 8,700 km (from the Mediterranean Sea to the Greenland Sea). This observation raised the question whether *H. dedrifera* actually represents a distinct taxonomic unit or a complex of (cryptic) species. Two other Atlanto-Mediterranean species of the same genus, *H. pruvoti* Topsent, 1896 and *H. racovitzai* Topsent, 1896 occurring in shallow-water or in caves and showing subtle differences with the deep-sea species were also included in the study. Due to their lack of mineral skeleton, verongid identifications are particularly challenging at the intraordinal and especially species level. But chemical analyses of *Hexadella* species sampled in shallow water and deep sea suggested the production of different secondary metabolites at different depths (Morris and Andersen, 1989). In this study we used phylogenetic congruence criteria between the Folmer partition of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the D3–D5 region of the nuclear large ribosomal subunit (28S rDNA) and the second intron of the nuclear ATP-synthetase beta subunit gene (ATPS) to delineate the three European *Hexadella* species, and to reveal the presence of cryptic species within the genus. The ATPS marker was tested for the first time for its applicability in sponge species delimitation (CHAPTER III). This study is published in Molecular Phylogenetics and Evolution (2010, 56:104–114).

Now that cryptic *Hexadella* species were revealed by molecular markers, the next step was to search for phenotypic characters with diagnostic value to differentiate them and to make these species available to the scientific community (i.e., for further zoological and ecological studies). Indeed, while genetic information is clearly an active compound of today's systematic allowing to detect cryptic species, to shed light on true levels of diversity

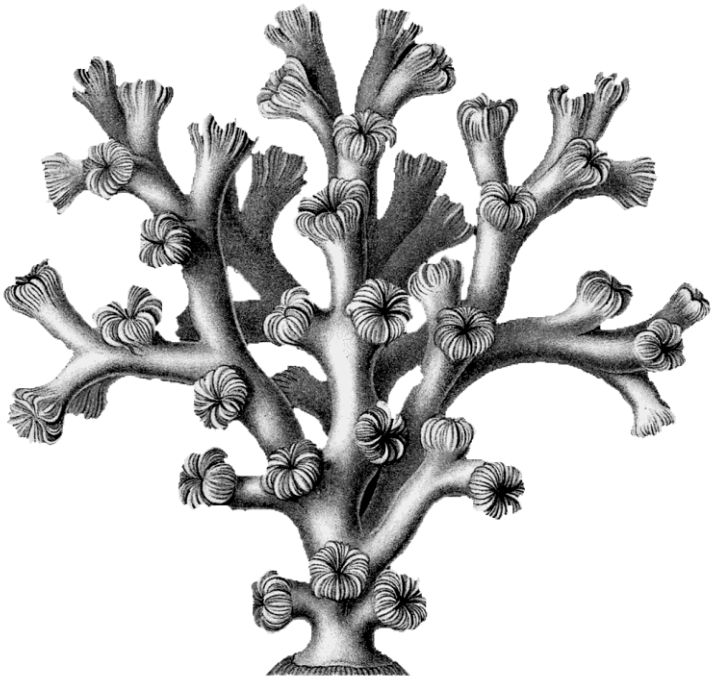
in the marine environment, and for reconstructing of taxonomic relationships in sponges, only a formal description will ensure access to the species and therefore make the best use of molecular discoveries. For feasibility reasons, we first focused on the newly highlighted shallow-water cryptic *Hexadella* lineages and sampled 27 new *Hexadella*-like specimens in the Mediterranean Sea. Future deep-sea campaigns (Bobeco IFREMER September-October 2011) allowing the sampling of fresh material may allow the taxonomic revision and description of the deep-sea cryptic *Hexadella* species. We combined phylogeny, morphology, cytology, chemistry, and toxicity in an integrative approach in order to revise the taxonomy of the shallow-water *Hexadella* species (CHAPTER IV). The COI gene of each individual was used to reconstruct the divergent genetic lineages of *Hexadella*. Starting from the general shape, consistent characters among color, surface structures, and distribution of openings of the aquiferous system, up to the ultra structure of the sponge cells and choanocyte-chamber types and sizes were investigated within and between each taxon. Special attention was directed to the cells with inclusions, which presence is emphasized in the original descriptions made by Topsent (1896). Eventually, we also provided species descriptions in order to make these species available to the scientific community. In this chapter, we promote the use of several scientific resources to delimit, discover, and describe meaningful natural species. This study is being prepared for publication.

Following another approach of integrated taxonomy, we used a combination of the COI fragment, partial 28S rDNA sequences and morphology to delineate small encrusting *Plocamionida* species, to detect the presence of cryptic species and to assess phylogenetic relationships within this group. Although widely distributed, *Plocamionida* remains a group of sponges that are notoriously difficult to identify because the intra- and interspecific character variation is not well understood. It has given rise to disagreements between taxonomic experts. In total, 46 specimens were retrieved from seven shallow to deep-water coral locations, crossing 3,000 km along the European margins (CHAPTER V). This study, showing and discussing the use of combining molecular and morphological information for the identification of sponge species has been published in PLoS ONE (2011, 10.1371/journal.pone.0016533).

Following the identification of divergent lineages within *Plocamionida* sp, a phylogeographic study within the most widespread *Plocamionida* species (i.e., *Plocamionida microcionides*) aimed to examine the connection between populations of CWC reefs associated species. These preliminary data are provided as an appendix to CHAPTER V, because a more extensive sampling would be necessary to support our tentative conclusions about phylogeography and connectivity. The issues related to the difficulties in obtaining enough sampling material from the deep sea in order to assess the connectivity and larval dispersal among deep-sea hotspot ecosystems, are discussed in a general discussion in CHAPTER VI. This last chapter also corresponds to the general conclusions of the thesis.

CHAPTER II

The distribution of Scleractinian Corals in the Bay of Biscay, NE Atlantic



Slightly modified from:

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Facies 54, 317-331

Abstract

Studies on the distribution of scleractinian corals in the Bay of Biscay were relatively numerous during the 19th and the first half of the 20th century. Yet, recent reports are scattered, sometimes unpublished, and therefore knowledge about the current coral occurrence in the area is limited. This study aims at compiling the available historical and more recently collected information on the occurrence of scleractinian corals in the Bay of Biscay. Data from two recent cruises are included and compared with previous explored coral sites from as early as 1830 up to 1995. A database of 347 records including 34 described species of scleractinians highlights that the slope of the Bay of Biscay is an important habitat for scleractinians. This could be expected due to the high topographic relief providing the necessary hard substrate and accelerated bottom current flow that corals require. Further exploration of the occurrence and ecology of corals in the area is recommended to support the conservation of cold-water coral reefs along the European margin.

Keywords: Cold-water corals; Scleractinia; Slope; Bay of Biscay; Biodiversity

Introduction

Cold-water coral distributions in both shallow and deep, bathyal settings are well studied along the European margins in the NE Atlantic Ocean, in the Mediterranean Sea, and recently in the Strait of Gibraltar (Zibrowius 1980, 1983, 1985; Rogers 1999; Roberts et al. 2003; Álvarez-Pérez et al. 2005; Taviani et al. 2005; Hall-Spencer et al. 2007). Cold-water coral assemblages have been recorded from the continental margin of the NE Atlantic more frequently than from any other place in the world (Roberts et al. 2006). Yet, the NE Atlantic margin consists of many different marine environments and scientific documentation about the distribution of corals in each segment varies considerably. Intensive exploration and investigation on the Norwegian margins (Mortensen et al. 1995; Hovland et al. 1998; Freiwald et al. 2002; Lindberg and Mienert 2005; Fosså et al. 2005) was certainly facilitated through the relatively shallow coral occurrences in some fjords and on various shelf banks, often < 400 m deep (Fosså et al. 2005). This has shed some light on the distribution, the biology and the geological settings of these coral ecosystems. The continental margin off Ireland and the UK, with its hundreds of coral banks in water depths between 650 and 1,000 m, recently emerged as a hotspot of integrated multidisciplinary research in Europe (Van Weering et al. 2003; Weaver et al. 2004; Huvenne et al. 2003, 2005; Foubert et al. 2005; De Mol et al. 2002, 2005; Wheeler et al. 2005, 2007; Roberts et al. 2006). Further south, the Bay of Biscay was already an authentic hotspot for oceanographic exploration since the end of the 19th century until the 1950's. In the first decades of the 20th century, this wide area was regularly visited by French biologists who made detailed investigations of the distribution and associated biodiversity of large coral reefs present in this area: the so-called 'massifs coralliens' (Joubin 1922a, b, 1923; Le Danois 1948). However, it is not known to which extent the available historical information represents the actual distribution of corals in the area, since the status of previously recorded coral locations may have completely changed (Hall-Spencer et al. 2007). Zibrowius et al. (1975) and Zibrowius (1980, 1985) already pointed to the presence of isolated patches of corals in the area with new observations of scleractinians from the French BIOGAS and INCAL expeditions to the Bay of Biscay. The latest species compilation of Cnidaria, including a list of Scleractinia, was published by Altuna (2006). Yet, information is scattered through various reports, while many scientific results remain unpublished resulting in a paucity of updated documentation for the Bay of Biscay (ICES 2003, 2004).

It is generally accepted that the Biscay region represents a suitable habitat for corals (Hall-Spencer et al. 2007). The presence of hard substrates is essential for coral reef formation both in shallow water and the deep sea (Dodge and Vaisnys 1977; Rogers 1990). Also a high bottom current flow, necessary for the delivery of food and larvae, as well as for the removal of waste and excess sediments (Grigg 1984) has been considered critical for reef development. Many observations indicate that scleractinians indeed occur in areas where the interaction between currents and topography can generate accelerated flow (Rogers 1999; De Mol et al. 2002; Freiwald 2003; Mienis et al. 2006). Further, it is likely that periodic oscillations in the vertical stratification of water density (internal waves) are important for corals. They indeed result in increased vertical and bottom mixing within the vicinity of the shelf break when interacting with the seabed. In this way, the influence of internal waves in controlling the particulate food supply by increasing resuspension of organic matter, has been described by Frederiksen et al. (1992). Finally, the presence of nutrient-rich waters[1] that stimulate high phyto-and zooplankton productivity, are providing a major food source for the corals (Freiwald et al. 2004). Given the notorious current-swept steep slope of the Bay of Biscay, cut by numerous canyons (Le Suavé et al. 2000; Bourillet et al. 2003, 2006; Zaragosi et al. 2006), it is expected that the water mixing over the slope may result in enhanced suspended material and indeed favours high concentrations of planktonic biomass which can sustain the coral growth.

This paper provides an overview of observations on scleractinian reef framework–forming species (stony corals) along the slope of the Bay of Biscay based upon historical and recent reports. Coral reports from the last two centuries collected in the Bay of Biscay are assembled in a database and mapped in a Geographical Information System (GIS) environment. The historical context of the successive exploratory surveys conducted along the slope of the Bay of Biscay, as well as the evolution of our knowledge on scleractinians are briefly addressed. The data from two recent research cruises -along the Banc de la Chapelle and in the Penmarc'h Bank area (PB) in the North, and the Cantabrian margin in the South- are also included. The disparity of the size and species assemblage of the reefs observed in the

[1] e.g., which may originate from upwellings

past compared to present-day reports and the wide occurrence of dead cold-water coral fragments are reported.

The main objective is to identify the recent past and present-day distribution and the diversity of scleractinians in the Bay of Biscay. This is of particular concern considering the low genetic differentiation of deep-water corals subpopulations along the NE Atlantic continental margin (Le Goff-Vitry et al. 2004; Le Goff-Vitry and Rogers 2005), suggesting a sporadic gene flow through larval dispersal over long periods of time. At a time where bottom fishing damage continues partly because coral-rich areas remain poorly mapped, it is essential to identify the potential and the importance of this region for the long-term protection of the European continental slopes.

Material and methods

Study area

The present study area covers the continental shelf, the shelf break, bathyal and abyssal zone of the Bay of Biscay (from 90 to 4,830 m), stretching from 43 to 49°N and from 0 to 11°W (Fig. 1a). The Atlantic margin in the Bay of Biscay can be subdivided into five geographic regions (Fig. 1a) -with in the North the French Celtic and the Armorican margins, and in the South the French Aquitaine margin and the Iberian Cantabrian and Galician margins. The Celtic and Armorican margin display a relatively broad shelf from the coast to the shelf break (wider than 200 km) and a steep slope with an average gradient about 2.86-5.15° (Le Suavé et al. 2000; Lallemand and Sibuet 1986) which extends from a depth of about 200-4,000 m towards the abyssal plain. The Cantabrian margin has a narrow shelf (5- 40 km wide) with a very steep continental slope (17°) between 500 and 4,500 m. A few marginal shelves (with a steep 10-12° slope) have been identified (Álvarez-Marrón et al. 1995). The most renowned of these Cantabrian marginal shelves, in relation to stony corals, is the 'Le Danois Bank' (Le Danois 1948). From the Norwegian margin to the Portuguese margin, the overall steep European continental slope is characterized by the presence of a multitude of canyons (Weaver et al. 2000). More than 80 canyons cut the 810 km of the Biscay slope (Le Suavé et al. 2000; Zaragosi et al. 2000; Canals et al. 2004). The hydrography of the Bay of Biscay is characterized by the presence of four main water masses: the North East Atlantic Central Water (NEACW) occupies the top 800 m of the water column; the large salinity

maximum at 800-1,200 m depth corresponds to the Mediterranean Outflow Water (MOW); between approximately 1,200 and 3,000 m, lies the North East Atlantic Deep Water (NEADW), including the Labrador Sea Water (LSW) and the Iceland-Scotland Overflow Water (ISOW; McCartney 1992). The LSW is recognized by a small salinity minimum at 1,800-2,000 m, and a small salinity maximum identifies the ISOW at ~2,600 m. The deepest water mass is the Lower Deep Water (LDW; McCartney 1992). By definition, the different water masses should tend to mix very slowly; however, hydrological measurements of the salinity, temperature and turbidity at the Celtic Margin along the Meriadzek Slope from 2,120 to 4,700 m depth (Vangriesheim 1985), pointed to overall water mixing at the seafloor in association with a layer of resuspended particulate material of the same thickness. The Celtic-Armorican margin has a wide continental plateau with little terrestrial input and intensive vertical mixing at the shelf break providing enhanced primary production. In contrast, the Cantabrian-Galician margin is characterized by a narrow shelf with strong continental input and by seasonal upwelling with high primary production. An important feature of the Northeast Atlantic is the slope current that flows along the continental slope from the Bay of Biscay to the Norwegian Sea (Pingree and Le Cann 1990; Ellett 1995). The MOW also flows poleward (Dickson et al. 1985).

Data origin

Historical records were extracted from the following sources: Joubin (1922a, b, 1923), Le Danois (1948), Zibrowius et al. (1975), Zibrowius (1980, 1985), Monteiro Marques and Andrade (1981), Álvarez-Claudio (1994) and Altuna (1995). Taxon, latitude, longitude, depth range and station identification were entered into a separate Microsoft Access database for each author, research vessel (R/V) and cruise. Arc Map 9.2 GIS was used to plot each dataset in a map to illustrate the distribution of cold-water corals in the area (Fig. 1b). Records are given in chronological order. Joubin (1922b) and Le Danois (1948), when reporting "white coral sites", did not make the distinction between the generally associated species *Madrepora oculata* (Linnaeus 1758) and *Lophelia pertusa* (Linnaeus 1758). The station lists extracted from these sources (including *Lophelia pertusa* and/or *Madrepora oculata*) do not make a distinction between the two species and hence are not described in detail.

The Banc de la Chapelle (BC) and the Cantabrian margin were revisited during respectively the R/V Victor Hensen VH-97 cruise in 1997 and the R/V Belgica GALIPOR cruise in 2004. During the VH-97 cruise (Freiwald and Henrich 1997), several Van Veen grab samples and dredges were collected in order to explore canyon heads near the BC and in the Penmarc'h Bank (PB) areas in water depths between 167 and 790 m (Fig. 2). The sample locations were based on the report of Le Danois (1948) in the area between 47°32'30"-47°51'50"N and 7°12'30"-7°25'W (BC) and in the area between 46°58'20"-46°58'30"N and 5°27'80"-5°28'W (PB). A 30 and 18 kHz single-beam echosounder was used for depth and morphologic control within the upper canyon heads and thalwegs[2]. The R/V Belgica 2004 GALIPOR cruise near the Cantabrian margin focused on an area between 44°05'00"-44°10'00"N and 7°10'-7°20'00"W, a sector highlighted by Le Danois (1948) and by Álvarez-Claudio (1994), as being rich in coral banks (see Fig. 1b). The area was surveyed with a Simrad E1002 multibeam echosounder and sampled with box cores (Foubert et al. 2004). Box cores were taken with a type NIOZ boxcorer, 30 cm diameter. These box cores allowed detailed biological sub-sampling for faunal analysis (cold-water corals and associated fauna) and the records were added to the general database and coral distribution map.

[2] i.e., the deepest inline within a valley

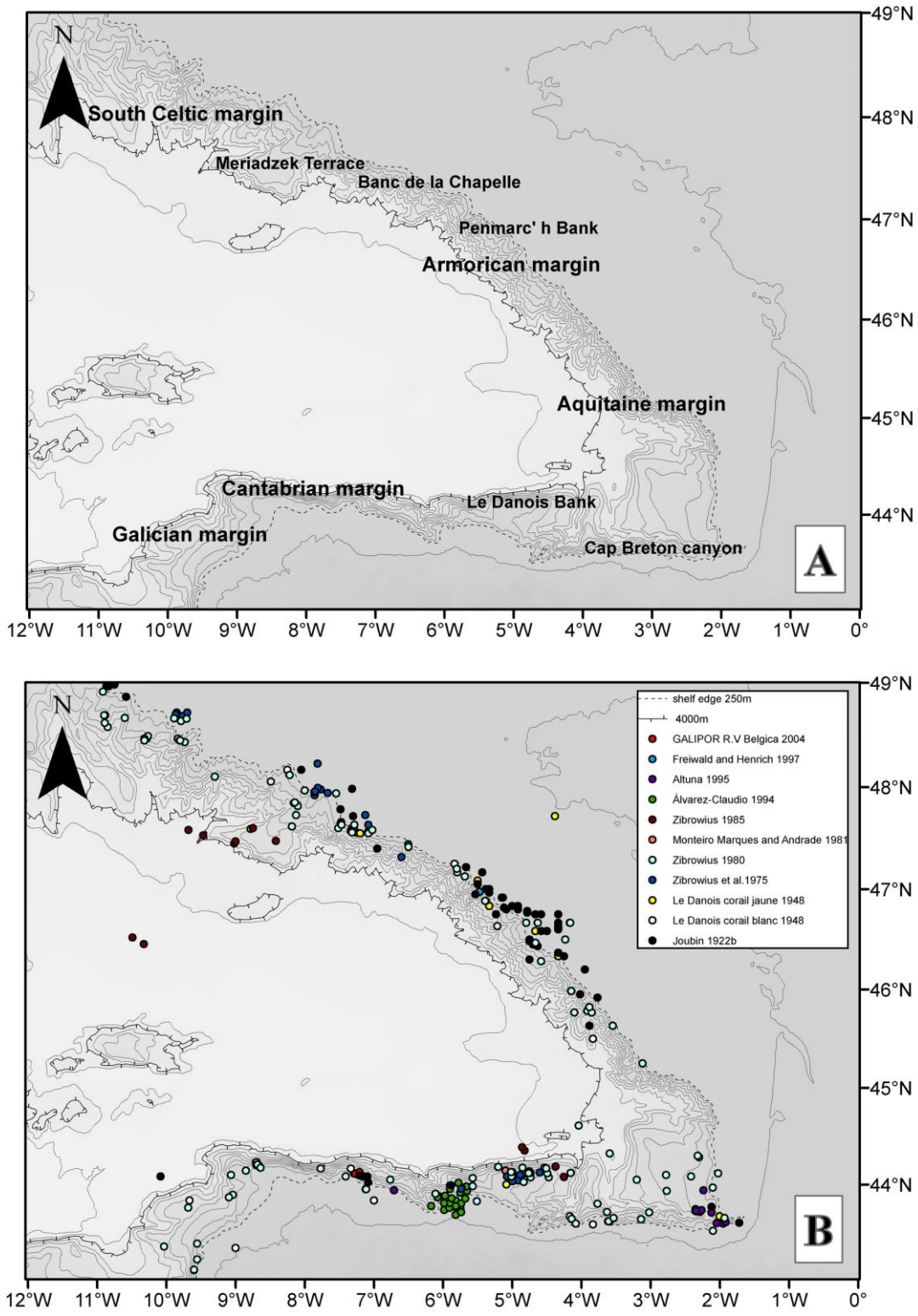

Fig. 1a The continental margin of the Bay of Biscay with geographic names used in the text. The shelf edge is on average at 250 m (dashed line) and the foot of the continental margin is at 4,000 m water depth (crossed line). Spacing of contour lines is 500m. **b** Findings of cold-water scleractinians cluster along the continental margin in the Bay of Biscay. The map is modified from Sibuet et al. (2004).

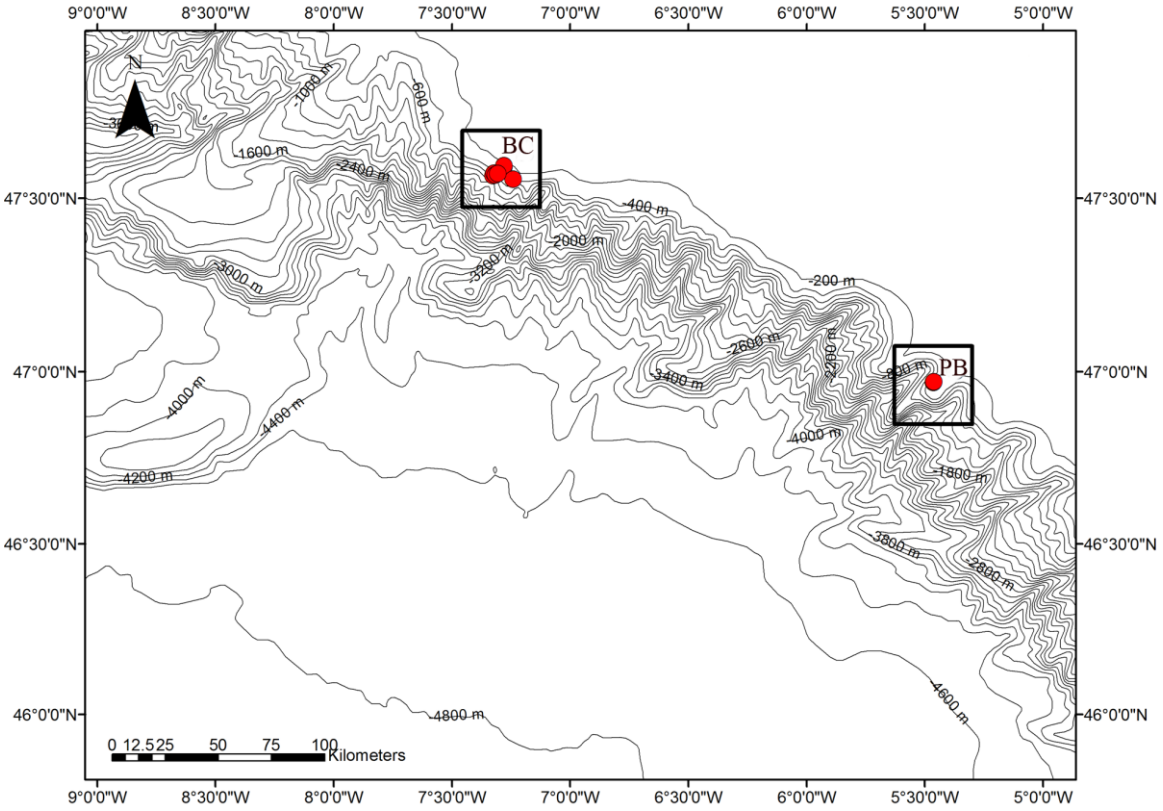

Fig. 2 Deep-water coral samples in the Banc de la Chapelle (BC) and Penmarc'h Bank area (PB), collected during the R/V Victor Hensen cruise VH-97.

Results

Historical data on scleractinian corals in the Bay of Biscay

In this section, an overview is given on the oceanographic campaigns realized in the Bay of Biscay from 1830 to 1995 as well as on the evolution of our knowledge on scleractinians from this area during this period. Cold-water coral research published up to now is presented in Table 1 and Figure 1b, indicating depth and reported species. The authors provide a more detailed compilation of species, geographic coordinates, depth and their station codes on the FACIES web site (see Electronic supplementary material). Although the list is not exhaustive, it clearly demonstrates the wealth of data on the distribution of corals in the Bay of Biscay, as well as the extent of the research activities taken in this area. Special attention will be given to major biogeographic areas where cold-water corals were repeatedly reported in the Bay of Biscay.

Historical overview of campaigns in the Bay of Biscay

During the end of the 19th century and the first half of the 20th century, the Bay of Biscay was explored by the most eminent oceanographers. Audouin and Milne Edwards conducted in 1830 the first oceanographic dredge surveys along the French coasts at different water depths (Le Danois 1948). The first deep-sea research campaigns in the Bay of Biscay began in 1870 with the British expeditions of the H.M.S. Porcupine (Duncan 1870, 1873, 1878) followed from 1890 to 1896 by the expeditions of the H.M.S. Research in the North of the Bay of Biscay and in 1906 of the H.M.S. Huxley (Hickson 1907) on the Little Sole and the BC (Le Danois 1948). The French government provided the R/V le Travailleur (1880-1882; De Folin 1887), the R/V le Talisman (1883) and then the R/V Caudan (1896) to the scientific community represented by Milne-Edwards (1881a, b), Roule (1896), Marion (1906) and Gourret (1906).

In 1885, the Prince of Monaco Albert I began a series of expeditions in the Atlantic. His first oceanographic campaign took place on board of the R/V l'Hirondelle (1886-1887) along the French coasts and in the Bay of Biscay (Jourdan 1895). On board of the R/V Princesse-Alice (1891 to 1896), he explored the entrance of the English Channel and returned in the Bay of Biscay. It is onboard of the R/V La Princesse–Alice II (1903 and 1904) that he sampled in total 150 stations in the Bay of Biscay and then the waters around Belle-Isle (1910). He explored for the last time the Bay of Biscay on board of the R/V Hirondelle II (1912-1914). The deep-sea corals collected during a series of campaigns between 1893-1913 were studied by Gravier (1915, 1920). Finally, in 1910, the Norwegian R/V Michaël–Sars, with John Murray, carried out a small expedition in the Bay of Biscay.

With the aim to produce a map of cold-water coral reef occurrences in the NE Atlantic, Joubin (1922a, b, 1923) enlisted fishermen from the ports of Lorient, La Rochelle and Arcachon in France to report on the sites where they encountered coral fragments in their nets. The zoologist Le Danois, first in collaboration with Charcot on board of the R/V Pourquoi-Pas? (1912- 1914), then on board of the R/V Perche in 1920, the R/V Tanche from 1921 to 1928, and finally the R/V President-Theodore-Tissier from 1934 to 1939, sampled

hundreds of stations along the French and Spanish coasts. During these investigations, he discovered the bank now bearing his name, Le Danois Bank.

During the following 30 years, the Bay of Biscay only received occasional attention. Samples and collections obtained from the expeditions on board of R/V Job ha Zélian (1963-1972), R/V Thalassa (1967- 1973), R/V Jean Charcot "Gestlante 2" 1967, R/V Jean Charcot "Noratlante" 1969, R/V Jean Charcot "Hespérides" 1976, R/V Walther Herwig 1974-1975 and R/V Sarsia 1954- 1976 were studied by Zibrowius et al. (1975) and Zibrowius (1980). Zibrowius et al. (1975) reported and described numerous stations with scleractinians in the NE Atlantic, including the Bay of Biscay. Stations from the Hespérides 1976 cruise off the Atlantic coast of the Iberian Peninsula were additionally reported by Monteiro Marques and Andrade (1981).

More intensive bathyal and abyssal research was resumed in the area in the framework of the French BIOGAS (Biology Gascogne) program, running from 1972 to 1981 (Laubier and Monniot 1985). Several selected stations within the northern and southern Bay of Biscay were visited during a total of 12 cruises on a variety of vessels including La Perle, Jean Charcot, Cryos, le Noroit and Capricorne (Zibrowius 1985). From 1987 to 1990, a French-Spanish project was conducted in the Cap Breton canyon (SE Biscay margin) on board of the Côte de Aquitaine (CNRS; Sorbe 1990). Two bathymetric zones were selected at respectively 70-400 m and 500-1000 m in order to follow up the investigations of Le Danois (1948). Altuna (1994, 1995) studied the bathyal Scleractinia from this campaign and summarized the state of knowledge on this taxon in the area. Finally, 25 stations were sampled during the 1987 COCASE cruise (Central Cantabric Oceanographic Cruise) in a small area of the Cantabrian margin, studied by Álvarez-Claudio (1994).

Evolution of the knowledge on Scleractinians in the Bay of Biscay

The majority of knowledge on corals reported from 1870 to 1920 (Duncan 1870, 1873, 1878; Milne-Edwards 1881a, 1881b; De Folin 1887; Jourdan 1895; Roule 1896; Marion 1906; Gourret 1906; Hickson 1907; Gravier 1915, 1920) is reviewed in Zibrowius (1980).

Joubin (1922a, b, 1923) mainly investigated the distribution of the two white coral species *Lophelia pertusa*, which was at that time still called *Lophohelia prolifera*, and *Madrepora oculata* called *Amphihelia oculata*. Also information on the coral species *Dendrophyllia cornigera* (Lamarck 1816) and *Dendrophyllia ramea* (Linnaeus 1758) were provided. He reviewed a total of 70 cold-water coral reef reports in the Bay of Biscay and described massive cold-water coral reefs so large and dense that fishermen fishing in these waters were afraid to damage their nets.

Le Danois (1948) also described the distribution of *Lophelia pertusa* and *Madrepora oculata*, while he recognized them as the two main reef forming species. Also *Desmophyllum dianthus* (Esper 1794), *Caryophyllia smithii* (Stokes and Broderip 1828) and *Flabellum alabastrum* Moseley, 1873 are often found associated with these framework-builder species (Le Danois 1948). Furthermore, the yellow coral *Dendrophyllia cornigera* was mentioned in this work as well. Usually forming low patches with a mean size of about 30 cm, this species occurred in large quantities within large patches of white corals. Among some isolated patches, Le Danois further identified five sites where the density of these species was high enough to form coral reefs along the margins of the Bay of Biscay: the "Massif du Banc de la Chapelle" (a surface of about 3500 km^2), the much smaller "Massif de la Grande Vasière" (south of Armorican Margin, with a length of 180 km), the "yellow coral" "Le Danois Bank" or "Massif Cantabrique", the "Massif Galicien de l'Est" (situated about 7°W) and the "Massif Galicien de l'Ouest" (stretching from 9°00' to 9°40').

Zibrowius et al. (1975) reported eight scleractinian species associated with the polychaete *Lumbriconereis flabellicola* (Fage 1937). In 1980, he reported 34 coral species from the Bay of Biscay including shallow-water species; in 1985, he reviewed the collections from the more recent cruises conducted in the Bay of Biscay (Programmes BIOGAS and POLYGAS, INCAL) and reported 11 species of scleractinians (Zibrowius 1985). The coral fauna from station 1 BIOGAS (1,920-2,350 m, northern Biscay margin) was characterized by a very high abundance of *Caryophyllia ambrosia* Alcock 1898 (2,000 to 3,000 individuals collected in one trawl) and *Premocyathus cornuformis* (Pourtalès 1868) and by the lower abundance of *Stephanocyathus nobilis* (Moseley 1873), *Flabellum alabastrum* Moseley 1873

(about 200 living individuals) and *Flabellum angulare* Moseley 1876. Zibrowius (1985) identified this coral assemblage as typical for soft bottoms. He described BIOGAS station 6 (1,894-2,430 m, southern Biscay margin) as a site of harder substrates where typical hard-bottom species were collected. Grasshoff (1981[3]a, b) already concluded the same through the study of other groups of Anthozoa, mainly Octocorallia. At the deepest stations (>4,000 m), a single species was collected, *Fungiacyathus marenzelleri* (Vaughan 1906).

[3] Grasshoff 1981 should be Grasshoff 1982

Table 1 Scleractinian species reported from the Bay of Biscay outer shelves, canyons and slopes based on Cairns and Chapman (2001) and few other sources cited in the text with depth ranges, numbers of samples and information on coloniality (*C* = colonial, *S* = solitary)

Species	**Depth [m]**	***n***	**Coloniality**
Anomocora fecunda (Pourtalès, 1871)	1000	1	C
Balanophyllia cellulosa Duncan, 1873	137-463	5	S
Balanophyllia thalassae Zibrowius, 1980	380-1150	12	S
Caryophyllia abyssorum Duncan, 1873	600-1340	13	S
Caryophyllia ambrosia Alcock, 1898	1520-2940	8	S
Caryophyllia atlantica (Duncan, 1873)	1107-1470	6	S
Caryophyllia calveri Duncan, 1873	200-1050	7	S
Caryophyllia cyathus (Ellis and Solander, 1786)	695-760	1	S
Caryophyllia sarsiae Zibrowius, 1974	500-1100	7	S
Caryophyllia seguenzae Duncan, 1873	910-2100	24	S
Caryophyllia smithii Stokes and Broderip, 1828	118-468	28	S
Deltocyathus conicus Zibrowius, 1980	1100-2430	4	S
Deltocyathus moseleyi Cairns, 1979	532-1372	9	S
Dendrophyllia alternata Pourtalès, 1880	450-688	2	C
Dendrophyllia cornigera (Lamarck, 1816)	50-620	36	C
Desmophyllum dianthus (Esper, 1794)	310-2350	25	S
Eguchipsammia cornucopia (Pourtalès, 1871)	330-960	7	C
Enallopsammia rostrata (Pourtalès, 1878)	915-2430	10	C
Flabellum alabastrum Moseley, 1873	800-2430	7	S
Flabellum angulare Moseley, 1876	1884-3800	8	S
Flabellum macandrewi Gray, 1849	790-930	3	S
Fungiacyathus fragilis Sars, 1872	910-1810	3	S
Fungiacyathus marenzelleri (Vaughan, 1906)	1884-4825	8	S
Javania cailleti (Duchassaing and Michelotti, 1864)	1200-2430	4	S
Lophelia pertusa (Linnaeus, 1758)	150-2000	102	C
Madrepora oculata (Linnaeus, 1758)	150-2000	86	C
Paracyathus pulchellus (Philippi, 1842)	121	1	S
Premocyathus cornuformis (Pourtalès, 1868)	700-2350	5	S
Solenosmilia variabilis Duncan, 1873	676-2430	10	C
Stenocyathus vermiformis (Pourtalès, 1868)	450-960	9	S
Stephanocyathus crassus (Jourdan, 1895)	476	1	S
Stephanocyathus moseleyanus (Sclater, 1886)	910-1570	14	S
Stephanocyathus nobilis (Moseley, 1873)	1430-2430	11	S
Vaughanella concinna Gravier, 1915	1175-2430	7	S

Álvarez-Claudio (1994) reported 1,100 cold-water coral specimens in a depth range from 50 to 1,347 m, belonging to 15 scleractinian species and 5 families. The analysis of the scleractinian species richness in the small sampling area of Cantabrian margin showed that only a few scleractinian species, such as *C. smithii*, settled on soft sandy shelf-bottom substrates. On the contrary, in deeper areas where the slope was less steep and bottoms were composed of very fine sand and mud, the scleractinian fauna was more diverse, comprising 5 to 7 species within *Lophelia pertusa* and *Madrepora oculata* banks. Álvarez-Claudio (1994) reported *C. smithii* (depth 146-468 m), *C. abyssorum* Duncan 1873 (depth 702-1,189 m) and *Lophelia pertusa* (depth 702-1,347 m) as the most commonly represented species along the Cantabrian margin and *Dendrophyllia cornigera* as the most shallow occurring species (50 m).

Altuna (1995) found seven species in total. He reported *Fungiacyathus fragilis* Sars 1872 for the first time in the Bay of Biscay. Numerous colonies and fragments of *Lophelia pertusa* and *Madrepora oculata* were obtained at 948 m water depth. *C. seguenzae* Duncan 1873 was also very frequently present in some stations.

According to the biogeographic analysis of Cairns and Chapman (2001), who compiled all data available at that time, a third of the scleractinian species that are known to occur in the two deep-water coral provinces embracing the Bay of Biscay -Province 1 from North Sea[4] to northern Bay of Biscay; and Province 2 from the southern Bay of Biscay- are endemic to the eastern Atlantic, while the others are amphi-Atlantic. Table 1 lists 34 scleractinian species collected from the Bay of Biscay in about 350 stations (see Fig. 1b). Eight species are colonial (23.6 %) and were present on most sampling sites. *Lophelia pertusa* was encountered on 102 (29.1 %) stations, followed by *Madrepora oculata* on 86 (24.6%) and *Dendrophyllia cornigera* on 36 (10.3 %). Amongst the solitary corals, *C. smithii* (28 stations, 8 %), *Desmophyllum dianthus* (25 stations, 7.1 %) and *C. seguenzae* Duncan 1873 (24 stations, 6.9 %) represent the most commonly found species (Table 1). A detailed account on species, depth ranges and stations is provided species-wise and can be downloaded from the journal's online documentary website (see Electronic supplementary material).

[4] The region actually ranges from Ireland to northern Bay of Biscay

New data

The grab samples (Fig. 2) on the shallow sandbank-dominated top of the BC area, yielded quartz sands rich in the solitary scleractinian *C. smithii*. In the deeper canyon heads, grab sampling often failed due to rough seabed morphology. Dead colonies of *Lophelia pertusa, Madrepora oculata* and *Desmophyllum dianthus* occurred from 340 (few) to 790 m (abundant). No living scleractinians were encountered. Further south, the outer Penmarc'h Bank was also explored (Fig. 2); but here, only Late Pleistocene *C. smithii* specimens were found which yielded calibrated U-Th[5] ages of 13,960±120, 13,060±240 and 11,170±180 years BP (Schröder-Ritzrau et al. 2005) at 240 m. The only live coral in this area were colonies of *Dendrophyllia cornigera* ("coral jaune") (Fig. 3). Along the Cantabrian margin, 3 out of 32 boxcores were filled with dead coral fragments. *Madrepora oculata*, numerous small pieces of *Lophelia* and one fragment of *Desmophyllum dianthus* were observed.

Fig. 3 *Dendrophyllia cornigera* collected alive from outer Penmarc'h Bank (VH-97, Station 320, 290 m depth)

[5] The Uranium-thorium (U-Th) is a radiometric dating technique frequently used to determine the age of calcium carbonate materials such as coral. The method in corals depends on the fact that coral skeletons form with very low Th/U ratios ($^{232}Th/^{238}U$ values of ~10^{-5}), which they inherit from seawater. The following ^{238}U-decay chain comprises ^{230}Th, whose activity generally raises with the age of the coral. The sample age is based on the difference between the initial ratio of Th/U and the one in the sample being dated (see Cobb et al., 2003).

Discussion

Worldwide, the aim is to have a better understanding of coral distributions and the factors that regulate them in order to manage these ecosystems more effectively (Cairns and Chapman 2001; Hall-Spencer et al. 2007; Bryan and Metaxas 2006, 2007; Davies et al. 2007). The NE Atlantic is the focus of the most extended historical literature available on deep-water corals. Out of 347 historical and new records, this study reports in total 34 species of scleractinians in the Bay of Biscay (see Table 1). The extraction of bathymetric information from these records shows a characteristic 'shallow-water' coral assemblage which is found on the shelf and incised upper canyon heads. From 10 to 500 m water depth, *Balanophyllia cellulosa, Caryophyllia smithii*, *Dendrophyllia cornigera* and *Paracyathus pulchellus*[6] form a characteristic neritic community (Fig. 4). A transitional group of corals exists on the outer shelf edge canyon heads at around 200 m and upper slope down to 2,000 m water depth. This group consists of *Balanophyllia thalassae, Caryophyllia calveri, Deltocyathus moseleyi, Desmophyllum dianthus, Dendrophyllia alternata, Eguchipsammia cornucopia, Stenocyathus vermiformis, Stephanocyathus crassus*[7]*, Lophelia pertusa* and *Madrepora oculata* (Fig. 4). The latter two framework-building species show a wide depth range from 200 m down to 2,000 m; however the majority of findings are in the shallow canyon heads (200-400 m depth interval). Corals confined to the upper slope from 500 to 3,000 m are *Anomocora fecunda* (1,000 m), *C. abyssorum* (600-1,400 m), *C. ambrosia* (1,600-3,000), *C. atlantica* (1,200-1,500), *C. cyathus* (700-800), *C. sarsiae* (500-1,100 m), *C. seguenzae* (1,000-2,100 m), *Deltocyathus conicus* (1,100-2,500 m), *Enallopsammia rostrata* (1,000-2,500 m), *Flabellum alabastrum* (800-2,500 m), *Flabellum macandrewi* (800-1,000 m), *Fungiacyathus fragilis* (1,000-1,900 m), *J. caileti* (1,200-2,500 m), *Premocyathus cornuformis* (700-2,400 m), *Solenosmilia variabilis* (700-2,500 m), *Stephanocyathus moseleyanus* (1,000-1,700 m), *Stephanocyathus nobilis* (1,500-2,500 m) and *V. concinna* (1,200-2,500 m). True deep bathyal corals are *Flabellum angulare* (1,900-3,900 m) and *Fungiacyathus marenzelleri* (1,900-4,900 m; Fig. 4).

[6] *Stephanocyathus crassus* should be added there

[7] *Stephanocyathus crassus* should be removed here

Based on the reports of Joubin (1922a, b, 1923), Le Danois (1948), Zibrowius et al. (1975), Zibrowius (1980, 1985), Monteiro Marques and Andrade (1981), Álvarez-Claudio (1994) and Altuna (1995) scleractinian corals appear to cluster in several key regions: the Meriadzek Terrace (MT), the BC, the Aquitaine margin, the Cap Breton Canyon, Le Danois Bank and the Cantabrian-Galician margin (Fig. 1b). The boxcore samples acquired both from the BC area in 1997 and from the Cantabrian-Galician margin in 2004 contained a large amount of dead fragments and coral rubble rather than pieces of living corals. Yet, the same coral reef building species as the ones described by Le Danois (1948), i.e., the triad *Lophelia pertusa*, *Madrepora oculata*, *Desmophyllum dianthus* were retrieved. A few live specimens of *Dendrophyllia cornigera* were observed in the PB (Fig. 3). However, the sampling intensity was certainly not enough to formulate a final statement on the presence of healthy corals, both in the BC-PB and the Cantabrian-Galician margin. Moreover, the steep Biscay slope proved difficult to be sampled. Some coordinate shifts from the ones provided by Le Danois were observed in the Cantabrian-Galician margin. The source of inaccuracy in estimating the distribution of *Lophelia pertusa* in this region may lie in the combination of the very steep topography of the slope, as well as in the randomly located transects, relative to isolated occurrences of these reefs.

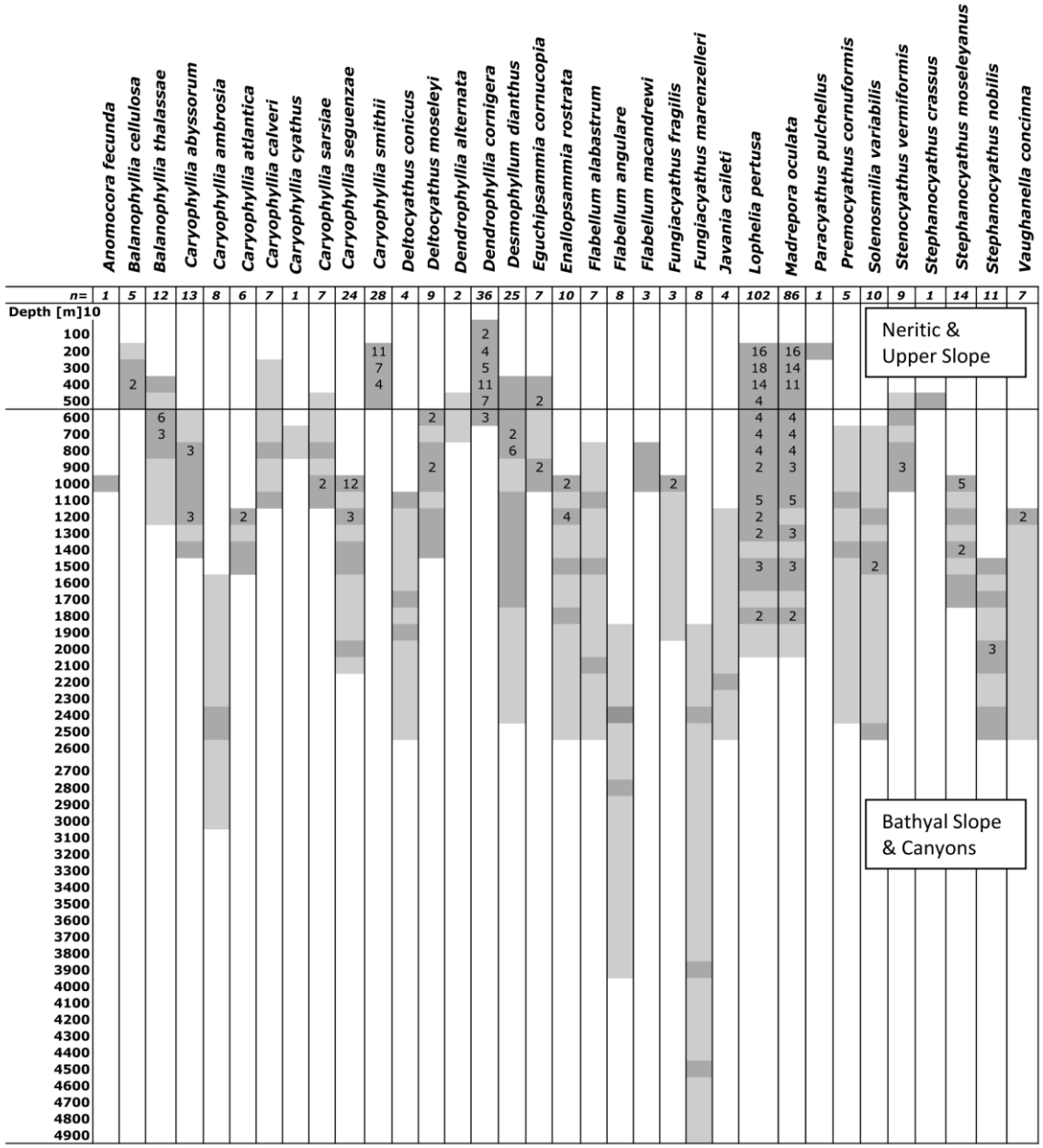

Fig. 4. Bathymetric ranges of 34 Scleractinia in the Bay of Biscay grouped in 100-m-intervals. Coding of bars: *dark grey* -coral sample, *bright grey* -interpolated, *numbers in bars* -frequency of coral samples from the given depth interval[8]

[8] n= number of samples reported per species. Dark grey corresponds to a coral sample in a defined 100-m depth range (i.e., *Balanophyllia thalassae* reported at 380 m was plotted at the interval depth of 400 m), while bright grey corresponds to the report of a coral sample which depth overlaps two or more 100-m interval (i.e., *Balanophyllia thalassae* reported at 860-1150 was plotted in bright grey in the following intervals: 900-1000-1100-1200). In case where the sum of the samples in bars does not match the 'n', it is due to some interpolated samples within the depth range highlighted. Gaps between plotted depths were also interpolated in bright grey.

It is likely that localized regions characterized by stronger bottom currents exist that may support the development of coral colonies and their associated assemblages in the area. There are few direct measurements of currents in the Bay of Biscay. However, the range of bottom current velocities can vary from 19.7 cm s^{-1} at a shallow (2,120 m) to 4.6 cm s^{-1} at deep (4,700 m) locations at the northern Bay of Biscay (Vangriesheim 1985). These data suggest bottom currents greater than 15 cm s^{-1} at shallower depths (<1,500 m), where corals are found in high abundance. This velocity is indeed a likely threshold for resuspension of surface sediments in the NE Atlantic continental margin (Thomsen and Gust 2002). Ripple marks were also observed on the MT indicating the presence of strong bottom currents (Laubier and Sibuet 1977). These bottom currents, strong enough to resuspend the sediment, may be related to internal tides (internal waves of tidal frequency) mainly occurring along the canyon axis (Gardner 1989). Indeed strong, localized internal tides in the Bay of Biscay (New 1988) result from a combination of a favourable stratification, a steep topography and strong barotropic tidal currents directed cross-isobath (Cartwright et al. 1980; Baines 1982; Pingree et al. 1986; Le Cann 1990; Pingree and New 1991). At the bottom within the canyons such as the Shamrock Canyon (north of MT), water mixing is responsible for resuspension of organic matter (Vangriesheim 1985). The canyons, mostly erosive and thus not filled with sediments (Le Suavé et al. 2000), probably induce locally strong currents and mixing which is essential for the corals to flourish. On the Nova Scotia margin, fishermen identified canyons as sites where dense gorgonian coral assemblages were thriving (Breeze et al. 1997). The significance of submarine canyons as coral habitats (Mortensen and Buhl-Mortensen 2005) may be due to their capacity to accumulate organic debris (Vetter and Dayton 1998), which may directly benefit suspension feeders. Submarine canyons are known to support high densities and diversity of megafaunal organisms (Hecker et al. 1980; Tyler and Zibrowius 1992), in greater abundance than in nearby slope areas (Hecker et al. 1980; Harrison and Fenton 1998; Vetter and Dayton 1998). Valuable reports are provided on higher fish occurrences in canyons and in relation to microtopography (Lorance et al. 2002).

Other examples document rich populations of stalked crinoids, *Diplocrinus whyvillethomsoni* (Conan et al. 1981), and pectinid bivalves (Schein 1989) on the bathyal slope of the Bay of Biscay. It is likely that this water mixing above the seabed results in enhanced levels of suspended material and favours higher concentrations of filter/suspension

feeders. Indeed, high faunal biomass occurs especially where internal waves result in an increase in suspended material (Rex 1981). Additionally, there appears to be a direct link between higher coral densities and high productivity levels in the overlying surface waters (White et al. 2005, 2007). The rapidly sinking seasonal blooms of surface primary production have been observed to have an effect on the reproductive biology of benthic invertebrates (Billett et al. 1983; Rice et al. 1991; Waller and Tyler 2005). A mass seasonal deposition of aggregated phytodetritus to the ocean floor was revealed for the first time at the Porcupine Seabight (Rice et al. 1991). It was also observed at a variety of other continental margin sites in the NE Atlantic, including the carbonate mounds in UK waters (Rockall Trough; Kenyon et al. 2003) and on the Galicia Bank (Duineveld et al. 1994[9]). Each year from May to September, the Celtic-Armorican shelf break exhibits a summer surface cooling of 0.5-1.5 °C (Dickson et al. 1980; Pingree et al. 1982), coupled to high chlorophyll levels (Pingree et al. 1986) and to an augmentation of new production. A seasonal recurring upwelling of cold waters is thought to be mainly due to the propagation of exceptionally large internal waves, generated by the interaction of strong barotropic tidal currents with the steep shelf break topography (Dickson et al. 1980; Pingree et al. 1986). On the Cantabrian margin, the surface currents generally flow eastwards during winter and spring and change westwards in the summer. These changes in the direction of currents produce seasonal coastal upwelling. A combination of (1) a high production in the surface waters (Dickson et al. 1980; Pingree et al. 1982), (2) the availability of suitable seabed surfaces for reef formation, and (3) favourable hydrodynamic conditions (New 1988; Le Cann 1990) to produce enhanced bottom currents and to promote the transport of organic material to the seafloor is probably responsible for the occurrence of a dominant fauna of filter and suspension-feeders in the Bay of Biscay.

Besides cold-water corals, also a high diversity and abundance of associated species assemblages were found in the Cantabrian-Galician region. Dead shells of the bivalves *Spondylus gussoni* (Costa, 1829) and *Lima marioni* (Fischer, 1882) were identified in large quantities. Echiurids (*Bonellia viridis* Rolando, 1821), polychaetes (*Eunice* spp.), molluscs (*Limopsis aurita* Brocchi, 1814), *Delectopecten vitreus* (Gmelin, 1791), crustaceans (*Munida sarsi* Huus, 1935), and many commercial fish are reported in a high quantity along the

[9] Duineveld et al. 1994 should be Duineveld et al. 2004

Cantabrian-Galician margin. Although no species is actually stated as being restricted to cold-water corals, these species are frequently found associated with cold-water corals (Jonsson et al. 2004; Freiwald et al. 2004; López Correa et al. 2005; Henry and Roberts 2007). A unique highly diverse and abundant suprabenthic community was also observed on Le Danois Bank; also a species of crustacean new to science was reported (Guerra-García et al. 2008). Finally, sponges are reported with high abundance (Le Danois 1948; Sánchez et al. 2002; Guerra-García et al. 2008). As major filter feeders, they may benefit from the same topographic and hydrological conditions required by stony corals; some[10] are used to discriminate on- and off-mound habitats in the NE Atlantic (Henry and Roberts 2007).

The presence of probably larger amounts of coral rubble beds in the area is yet a critical issue (i.e., on Le Danois Bank). Live corals indeed attract a huge international attention (Roberts et al. 2006); yet, dead and live coral reefs are inevitably associated with each other. Both are co-occurring on carbonate mounds along the continental margin of the NE Atlantic (Van Weering et al. 2003; Huvenne et al. 2005; Foubert et al. 2005; De Mol et al. 2002, 2005) and both participate to the reproductive cycle of coral reefs (Wilson 1979). Hard substrate on which to attach, such as coral rubble and dead fragments, is fundamental for any putative recolonization (Freiwald and Wilson 1998). The numerous dead cold-water corals may hence play a critical role for colonization by larvae from neighbouring isolated coral reefs in the Bay of Biscay (Roberts et al. 2006). In addition, where live cold-water corals mainly provide protection and even nurseries for some mobile species (Rogers 1999), dead stony corals have been observed to provide a substrate for an associated fauna which is even more diverse (Mortensen et al. 1995; Freiwald 2002). It is likely that coral skeleton, found in high quantity in the area, provide additional hard substratum for attachment of epifaunal organisms. Conversely, disturbance of coral remnants prevents the maintenance of an area free of resuspended sediment and may even provoke polyp suffocation. In shallow water, Dodge and Vaisnys (1977) have shown that extended dredging operations have had a destructive effect on coral communities. Damage in cold-water coral areas inflicted by significant coral by-catch when trawling is well documented in the Porcupine Seabight (Hall-Spencer et al. 2002; Grehan et al. 2005). Genetic analysis of populations in the Darwin mound

[10] The authors actually refer to some suspension feeders (hydroids), which are used to discriminate on-and off-mound habitats in the NE Atlantic.

province, NE Rockall Trough (Masson et al. 2003) revealed the least genetic diversity of any of the sites in the NE Atlantic (Le Goff-Vitry et al. 2004). Reproductive analysis studies also showed no reproductively active polyps in *Lophelia pertusa* taken from the Darwin mounds (Waller and Tyler 2005). Intense trawling areas were actually observed in this area (Hall-Spencer et al. 2002). It was hypothesized that the damage from these operations may impede *Lophelia pertusa* from reaching the size necessary for gametogenesis (Waller and Tyler 2005). Many scleractinian colonies indeed have to reach a certain size before acquiring reproductive maturity (Szmant 1986), while stress may reduce reproductive output, and even cause death in some cases (Brown and Howard 1985). A small, but non-permanent deep-sea fishery (e.g., for *Hoplostethus atlanticus* Collett 1889, commonly named orange roughy) is established off France in the Bay of Biscay (Koslow et al. 2000). Fishermen, principally looking for orange roughy, mainly within the vicinity of canyon heads in the northern part of the Bay of Biscay (47° N/ 49°N), report living *Lophelia pertusa* by-catch until 1,100 m (Le Guilloux personal observation).

Anthropogenic impact on this area, e.g. in terms of fishery has not yet been addressed so far. The consequences may be significant because the fishing activity occurs within a narrow continental margin. Yet the steep topography could partially protect the coral communities by their inaccessibility for benthic trawling. However, it is also possible that many of the reefs of the Bay of Biscay may have been, like in other areas, obliterated by slumping and erosion (Gage and Tyler 1991). There has actually recently been an increase in evidence that deep-water reefs are fragmented (Rogers 1999; Waller and Tyler 2005). Yet localized regions of high coral abundance exist that may support cold-water coral recolonization. Genetic and phylogeographic analysis of deep-water corals in the NE Atlantic (Le Goff-Vitry et al. 2004; Le Goff-Vitry and Rogers 2005) suggest that *Lophelia pertusa* does not form a panmictic population. Instead, there is a high genetic differentiation between subpopulations in fjords and those offshore. Along the NE Atlantic continental margin, the genetic differentiation can be regarded as moderate suggesting sporadic, but not continuous, gene flow through larval dispersal over long periods of time. These last genetic analyses actually suggest that a certain genetic cohesion is maintained through larval dispersal over the slope of the Bay of Biscay. Consequently, the Biscay margin presumably acts as a semi-continuous habitat for larval dispersal of stony corals. The species distribution analysis of

Cairns and Chapman (2001) also highlights that the Bay of Biscay nicely fits into the NE Atlantic biogeographic cluster. The wide Bay of Biscay canyons and slopes may hence represent a stepping-stone for dispersal. Indeed, due to its central position within the European margins and in a scenario of a possible recolonization, the relative importance of the Bay of Biscay for the global distribution of cold-water coral reefs in the NE Atlantic is highlighted. The Bay of Biscay is a crucial and obligate transit route for cold-water coral colonization from or to the Mediterranean Sea and to the NE Atlantic. During interglacial periods, deep and intermediate-water flow is directed poleward along the NE Atlantic continental margins (Kenyon 1986), thus supporting a northward migration of deep-water corals.

Conversely, a southern retreat of corals and other shelly benthic invertebrate assemblages from northern to southern latitudes occurred during the transition from warm to cold climatic conditions. This phenomenon is evidenced by the immigration of a boreal fauna into the Mediterranean Sea, both in shallow and deep-water environments (Taviani et al. 1991; Schröder-Ritzrau et al. 2005). In this sense, the "boreal guests" in the Mediterranean Sea found a refugium to survive and to expand distribution back into the Atlantic when climatic conditions switched back to an interglacial mode. It is tempting to suggest, that Mediterranean deep-water coral dispersal into the NE Atlantic was supported by the oceanographic circulation that carried the genetic information from Gibraltar as far north as to the Barents Sea (De Mol et al. 2005). Establishment of water masses of different properties might have introduced larvae of the cold-water corals to the NE Atlantic and initiated the carbonate coral mounds in the Porcupine Seabight (De Mol et al. 2002, 2005). After the Last Glacial Maximum between 14 to 10 ka BP, corals began to recolonize the Bay of Biscay canyons and open slopes and settled on the pre-existing mounds in the Porcupine Seabight and Rockall Trough (Frank et al. 2005; Schröder-Ritzrau et al. 2005). Late Pleistocene scleractinians, 14-11 ka BP (Schröder-Ritzrau et al. 2005), as well as primnoid octocorals about 11 ka BP (Noé et al. 2007) recovered in the BC and the Cantabrian-Galician margin, offer new data to get a better insight into the migration pattern in space and time and the relation between the Atlantic and Mediterranean coral communities.

Conclusions

The compilation of historical information on deep-water habitats provided a wealth of information about scleractinian distribution in the Bay of Biscay. Considering the several cold-water coral rich areas as potential stepping-stones for larval dispersal, it highlights the need for additional visual surveying and mapping for cold-water corals in the area. Indeed at a time where more advanced seabed mapping and sampling tools are now more accessible than ever before, it is highly recommended to get deeper insight into the current state of cold-water corals over the whole Bay of Biscay. The Bay of Biscay is an integral sector of the European Margin, therefore better knowledge about corals in the area will help to make decisions on the management and the conservation of the deep-water coral environment along the European continental margins.

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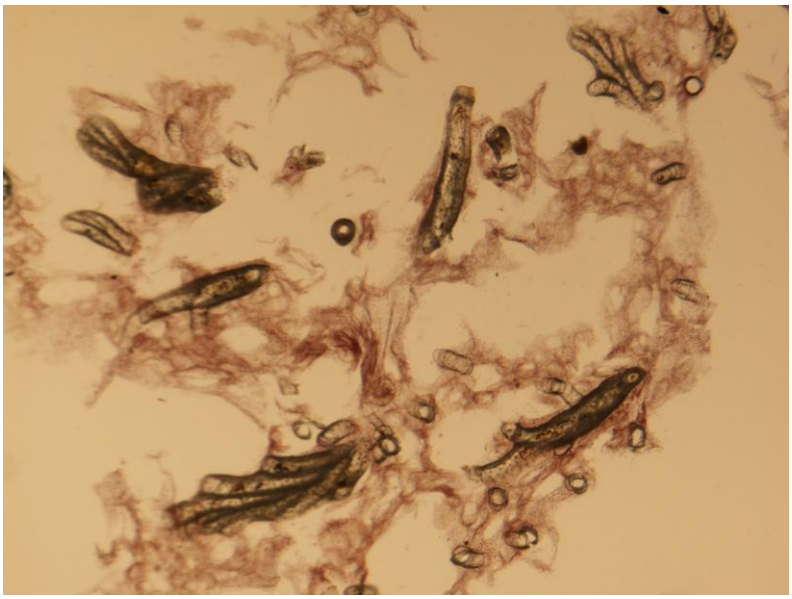
CHAPTER III

Species Boundaries and Phylogenetic Relationships between Atlanto-Mediterranean Shallow-water and Deep-sea Coral Associated *Hexadella* species (Porifera, Ianthellidae)



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Abstract

Coral reefs constitute the most diverse ecosystem of the marine realm and an increasing number of studies are focusing on coral species boundaries, distribution, and on processes that control species ranges. However, less attention has been paid to coral associated species. Deep-sea sponges dominate cold-water coral ecosystems, but virtually nothing is known about their molecular diversity. Moreover, species boundaries based on morphology may sometimes be inadequate, since sponges have few diagnostic characters. In this study, we investigated the molecular diversity within the genus *Hexadella* (Porifera, Demospongiae, Verongida, Ianthellidae) from the European shallow-water environment to the deep-sea coral ecosystems. Three molecular markers were used: one mitochondrial (COI) and two nuclear gene fragments (28S rDNA and the ATPS intron). Phylogenetic analyses revealed deeply divergent deep-sea clades congruent across the mitochondrial and nuclear markers. One clade contained specimens from the Irish, the Scottish, and the Norwegian margins and the Greenland Sea (*Hexadella dedritifera*) while another clade contained specimens from the Ionian Sea, the Bay of Biscay, and the Irish margin (*H.* cf. *dedritifera*). Moreover, these deeply divergent deep-sea clades showed a wide distribution suggesting a connection between the reefs. The results also point to the existence of a new deep-sea species (*Hexadella* sp.) in the Mediterranean Sea and of a cryptic shallow-water species (*Hexadella* cf. *pruvoti*) in the Gorringe Bank. In contrast, low genetic differentiation between *H.* cf. *dedritifera* and *H. pruvoti* from the Mediterranean Sea was observed. All *Hexadella racovitzai* specimens from the Mediterranean Sea (shallow and deep) to the Atlantic formed a monophyletic group.

Keywords: Porifera; cold-water coral; COI; Nuclear intron; Partial 28S rDNA; phylogenetic resolution; Atlanto-Mediterranean.

CHAPTER III

Introduction

Coral reefs constitute one of the most diverse and vulnerable ecosystems of the marine realm (Hughes et al., 2003; Pandolfi et al., 2003). Recent evidence suggests that deep-sea coral ecosystems may compare in species richness and abundance to their shallow-water counterparts (Freiwald et al., 2004; Roberts et al., 2006; Cairns, 2007), and act as diversity hot-spots (Roberts et al., 2006; Lindner et al., 2008). Understanding the origin and evolution of this marine biodiversity is essential for its conservation and sustainable management (Palumbi, 2004). Species boundaries and processes that control the distribution of coral species are hence receiving increasing attention, from the shallow-water environment to the deep-sea (France and Hoover, 2002; Le Goff-Vitry et al., 2004; Reveillaud et al., 2008; Lindner et al., 2008). However, less attention has been paid to the species associated with the deep-sea coral reefs, despite the fact that they represent the highest biodiversity in these ecosystems (Roberts et al., 2006). Sponges constitute an important and dominant invertebrate group in hard-bottom benthic communities and play key roles in ecosystem functioning (Bell, 2008). Although ca. 200 Porifera species dominate the deep-water coral reef ecosystems, deep-water sponge species remain until today a barely explored taxon (e.g., Vacelet, 1969; Jensen and Frederiksen, 1992; Rogers, 1999; Longo et al., 2005; Van Soest et al., 2007a).

Sponges are a group with numerous but considerably plastic morphological traits. Characters such as texture, form, and coloration are not reliable for species delimitation as they are frequently influenced by environmental factors (Palumbi, 1984; Jones, 1984; Schönberg and Barthel, 1997; Carballo et al., 2006). Morphology of spicules and skeletal arrangement are traditionally the diagnostic characters to identify sponge species (Hooper and Van Soest, 2002). Unfortunately, spicule size and micromorphology can also be influenced by the environment (Palumbi, 1986; Maldonado et al., 1999; Bell et al., 2002). Consequently, taxonomic and systematic uncertainty may prevail, especially in species with a low number of informative characters (Klautau et al., 1999; Knowlton, 2000). Molecular markers repeatedly revealed cryptic species and proved valuable in delineating species boundaries in Porifera (Solé-Cava et al., 1992; Boury-Esnault et al., 1992, 1999; Klautau et al., 1994, 1999; Lazoski et al., 2001; Wulff, 2006; Blanquer and Uriz, 2007; Cárdenas et al., 2007). However, molecular studies in sponges from remote environments, such as the deep-sea, remain scarce. Knowledge on speciation within sponges from cold-water coral ecosystems has potentially great implications for the efficient conservation and economic management of this group

(Van Soest and Lavaleye, 2005). Moreover, peculiar life-history traits of sponges, such as a restricted larval dispersal (Maldonado, 2006), may induce high genetic structure (Duran et al., 2004; Calderón et al., 2007; Blanquer et al., 2009) making sponges a particularly interesting model group to determine the degree of connectivity between deep-sea reef populations.

In this study we examine the species boundaries and phylogenetic relationships between members of the genus *Hexadella* Topsent, 1896 (Order Verongida, Family Ianthellidae) collected along the European margins in both shallow-water and deep-sea habitats. Verongid sponges produce secondary metabolites with potential antithyroidic and antibiotic activities, and are therefore of particular interest for biotechnological applications (Wu et al., 1986; Bergquist and Cook, 2002; Erwin and Thacker, 2007; Erpenbeck and Van Soest, 2007). Chemical analyses of *Hexadella* species sampled in shallow-water and deep-sea suggested the production of different secondary metabolites at different depths (Morris and Andersen, 1989). However, due to their lack of mineral skeleton, verongid identifications are particularly challenging at the intra-ordinal and especially species level. Taxa are distinguished almost exclusively by the structure and arrangement of their spongin fibers (Bergquist and Cook, 2002), and in the case of fiberless species such as *Hexadella* spp. by the type and size of the choanocyte chambers. Taxonomists are thus left with very few morphological diagnostic characters (Topsent, 1896). Consequently, these and similar genera have been widely reported simply as ‘crustose sponges’ (Mortensen et al., 1995). Especially, *Hexadella dedritifera* Topsent, 1913 is a common species with a thin/soft growth form in deep-water coral ecosystems along the European margins. Fine crusts of *H. dedritifera* are found in the deep-sea on top of rocks, large sponges (e.g., astrophorids) or coral rubble in the Mediterranean Sea (Longo et al., 2005) and in the North East Atlantic (NEA: the Gulf of Cadiz, the Bay of Biscay, the Porcupine Seabight, Van Soest et al., 2007a; the Rockall Bank, Van Soest and Lavaleye, 2005; along the Norwegian margin and in the Greenland Sea, Topsent, 1913 and this study). However, such wide geographical distribution, over a distance of more than 8700 km (from the Mediterranean Sea to the Greenland Sea) raises the question whether *H. dedritifera* actually represents a distinct taxonomic unit or a complex of (cryptic) species. The two other Atlanto-Mediterranean species of the genus, *Hexadella pruvoti* Topsent, 1896 and *Hexadella racovitzai* Topsent, 1896 occur in shallow-water or in caves, although *H. pruvoti* has also been observed in deep water (J. Vacelet, personal communication). A subtle pink color distinguishes *H. racovitzai* from *H. dedritifera* and *H. pruvoti*, the latter two being bright yellow when alive and turning deep purple when taken out

of the water (aerophobic reaction). Furthermore, the deep-sea specimens of *H. dedritifera* have larger choanocyte chambers than both *H. racovitzai* and *H. pruvoti* (Topsent, 1913). With such subtle differences, it remains unclear whether *H. dedritifera* and *H. pruvoti* are two separate species or not.

Herein, we used phylogenetic congruence criteria between the Folmer partition of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the D3-D5 region of the nuclear large ribosomal subunit (28S rDNA) and the second intron of the nuclear ATP-synthetase beta subunit gene (ATPS) to delineate the three European *Hexadella* species, and to reveal the presence of cryptic species within the genus. The ATPS marker was tested for the first time for its applicability in sponge species delimitation.

Materials and methods

Sampling

Species of the genus *Hexadella* were collected at various locations throughout their distribution range (Table 1 and Fig. 1). The boundary between shallow water and deep sea was defined at a depth of 50 m, as previously used for corals (Cairns, 2007). Samples of *H. dedritifera* were collected with boxcores or Remote Operated Vehicle (ROV) during 7 deep-sea cruises (see Table 1). The deep-sea Mediterranean sample (180 m) collected in a canyon close to la Gabinière, Port-Cros, France could not be unambiguously identified because it formed some degenerating crusts when alive. Subsequent phylogenetic analyses (see below) revealed relationships to *H. racovitzai*. Three deep-sea specimens collected at the Ionian Sea location could not be unequivocally assigned to *H. dedritifera* due to a color variation when fixed in ethanol (dark green instead of dark violet). Hereafter, these three specimens will be referred to as *Hexadella* sp. Samples were preserved in absolute ethanol and stored at room temperature until further processing.

DNA extraction

Total genomic DNA was extracted from sponge tissue using the DNeasy Blood and Tissue Kit (Qiagen) following the instructions of the manufacturer. The standard protocol was optimized by adding a 25-min centrifugation step in the Savant Speed Vacuum System to eliminate ethanol prior to cell lysis, and increase final DNA yields. Amplifications by polymerase chain reaction (PCR) were performed in a total volume of 45 µl, with 5 µl 10x

PCR buffer (Qiagen), 1 µl $MgCl_2$ (25 mM), 1 µl dNTP (10 mM), 0.5 µl of BSA (10 µg/µl), 1 µl of forward and reverse primer (25 µM), 0.25 µl TopTaq DNA polymerase (Qiagen), and 1 µl of template DNA, and 34.25 µl of distilled water.

Amplification of COI fragment

PCR amplification of the 5' partition (Folmer et al., 1994) of the cytochrome c oxidase subunit I (COI) mtDNA was performed using the degenerated primers from Meyer et al. (2005) dgLCO 5'-GGT CAA CAA ATC ATA AAGAYA TYG G -3', and dgHCO 5'-TAA ACT TCA GGGTGA CCA AAR AAY CA-3' with PCR cycling parameters: 94 °C for 2 min, followed by 35 cycles of (94 °C for 40 s, 42 °C for 40 s, 72 °C for 60 s) and a final extension at 72 °C for 10 min. The nuclear tree topology was investigated for phylogenetic congruence with the mitochondrial tree topology. Therefore, representatives of each COI haplotype were sequenced for both the nuclear ATPS and 28S rDNA. A variable number of individuals (between 33% and 75%) were sequenced for each haplotype, depending on the haplotype frequency.

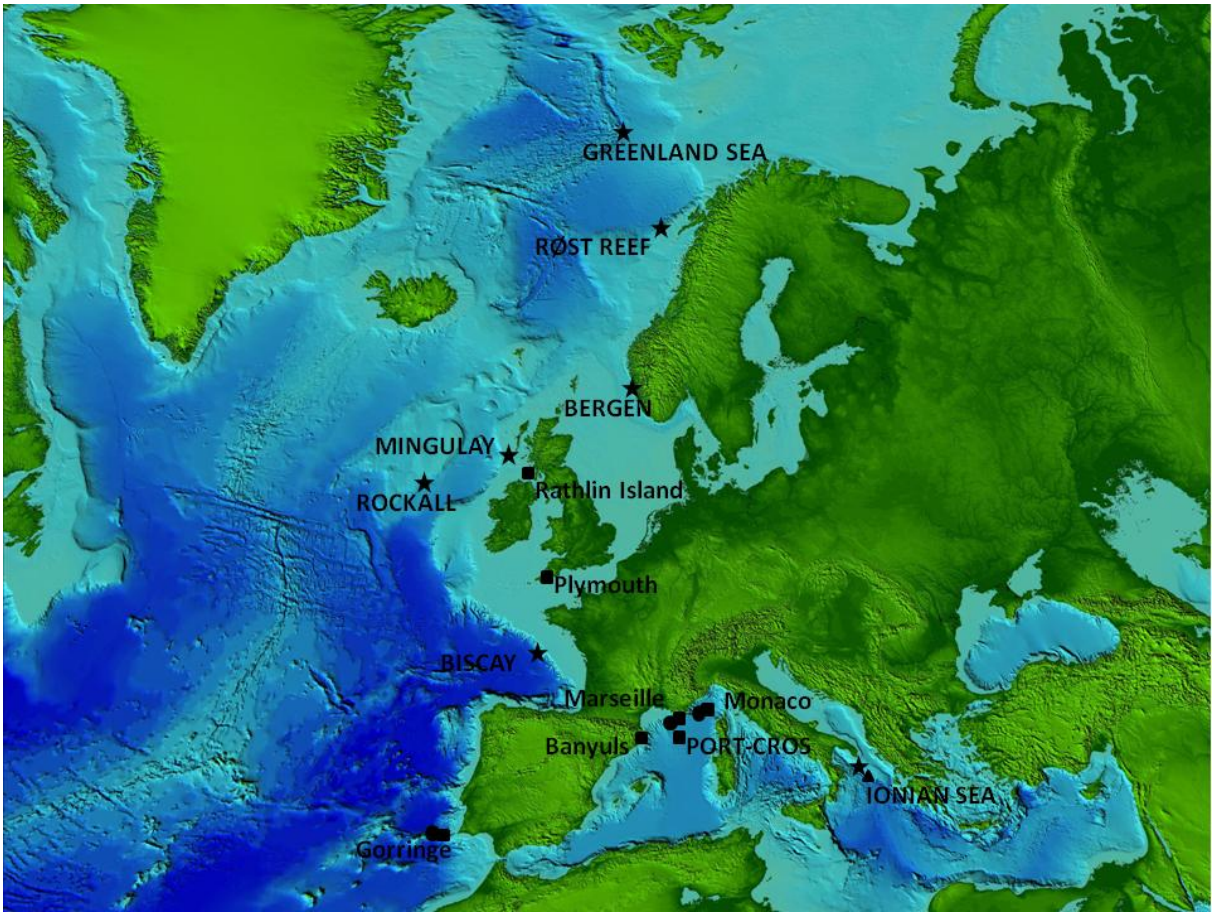

Fig 1. Map showing sampling localities of *H. dedritifera* (star shape), *H. pruvoti* (round shape) and *H. racovitzai* (square shape) [11]. For abbreviations of sampling localities see Table1. Map was provided by the project Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE).[12]

[11] *Hexadella* sp (triangle shape).

[12] Sampling localities are given in uppercase letters for deep-water samples (> 50 m) and in lowercase letters for shallow-water samples.

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Table 1. ***Hexadella dedritifera*** **(D),** ***Hexadella racovitzai*** **(R) and** ***Hexadella pruvoti*** **(P) specimens analyzed in the present study. Information regarding the sampling (region, localities, sampling method, coordinates, depth), number of individuals studied for each marker, and number of different haplotypes (No. h) is provided. Sampling location abbreviations are given in uppercase letters for deep-sea samples (>50m) and in lowercase letters for shallow-water samples.**

Species	Region	Localities	Deep-sea cruise, shallow water sampling (collector) or Museum	Lat.[N]	Lon.	Depth[m]	COI	No.h	ATPS	No.h	28S	No.h
H. dedritifera (D)	North East Atlantic	Ireland, Rockall Bank (ROC)	Moundforce 2004 (R/V *Pelagia*), BIOSYS/HERMES 2005 (R/V *Pelagia*)	55,45/55,50	-15,78/-16,11	575-773	7	3	7	2	4	4
H. dedritifera (D)	North East Atlantic	Norway, Rost reef (ROS)	ARK-XXII/1a 2007 (R/V *Polarstern*)	66,96/67,50	9,42/11,13	319-345	7	4	6	3	4	2
H. dedritifera (D)	Mediterranean Sea	Italy, Ionian Sea (ION)	Medeco 2007 (R/V *Pourquoi Pas?*)	39,56/39,61	18,43/18,50	561-649	7	1	2	1	3	1
H. dedritifera (D)	North East Atlantic	France, Bay of Biscay (BIS)	Biscosystem 2008 (R/V *Belgica*)	48,90/48,91	-5,32/-5,33	676	3	1	2	1	3	1
H. dedritifera (D)	North East Atlantic	Norway, Bergen (BER)	ZMA (Zoological Museum of Amsterdam)	60,30	5,10	100	2	1	1	1	2	1
H. dedritifera (D)	North East Atlantic	Scotland, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V *Pelagia*)	56,82	-7,37/7,39	128-137	2	1	2	1	2	1
H. dedritifera (D)	North East Atlantic	Greenland Sea, Schultz Seamount (GRE)	H2DEEP 2008 (R/V *G.O.Sars*)	73,95	7,71	688	2	1	1	1	2	1
H. pruvoti (P)	North East Atlantic	South Portugal, Gorringe Bank (gor)	Scuba diving (Joana Xavier)	36,51/36,71	-11,16/-11,56	39-42	2	1	2	1	1	1
H. pruvoti (P)	Mediterranean Sea	France, 3PP coral caveMarseille-Monaco (mam)	Scuba diving (Thierry Perez)	43,21/43,73	5,33/7,42	15-20	3	1	3	1	2	1
H. racovitzai (R)	Mediterranean Sea	France, 3PP coral cave Marseille-Monaco (mam)	Scuba diving (Thierry Perez)	43,21/43,73	5,33/7,42	15-20	3	2	1	1	2	1
H. racovitzai (R)	Mediterranean Sea	France, Banyuls (ban)	ZMA (Zoological Museum of Amsterdam)	42,50	3,13	35	1	1	1	1	1	1
H. racovitzai (R)	Mediterranean Sea	France, Port Cros (CRO)	MedSeaCan (R/V *Minibex*)	43,00	6,39	180	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	South Portugal, Gorringe Bank (gor)	Scuba diving (Joana Xavier)	36,51	-11,56	32	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	England, Plymouth, Outer Heybrook Bay (eng)	Scuba diving (Bernard Picton and Claire Goodwin)	50,31	-4,12	30	1	1	1	1	1	1
H. racovitzai (R)	North East Atlantic	Northern Ireland , Rathlin Island (ire)	Scuba diving (Bernard Picton and Claire Goodwin)	55,29	-6,25	30	1	1	1	1	1	1
Hexadella sp.	Mediterranean Sea	Italy, Ionian Sea (ION)	Medeco 2007 (R/V *Pourquoi Pas?*)	39,56/39,61	18,43/18,50	648	3	1	1	1	3	1

[13] The Grotte à Corail sampling locality, close to the Maire Island (Marseille) is erroneously referred to as the 3 PP cave.

Amplification of 28S rDNA fragment

PCR primers RD3A 5'-GAC CCG TCT TGA AAC ACG A-3' and RD5B2 5'- ACA CAC TCC TTA GCG GA-3' (McCormack and Kelly, 2002) were used for amplification of the D3-D5 fragment of the 28S rDNA gene under a temperature regime of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 20 s at 45 °C, 1 min at 72 °C and a final elongation of 10 min at 72 °C. Because PCR-amplification of some individuals was problematic, we designed *Hexadella* specific forward and reverse primers Hex28F (5'-CCG AAC AGG GTG AAG CCA GG-3', located 209 bp downstream of RD3A) and Hex28R (5'-TTACA CAC TCC TTA GCG G-3', located 1 bp downstream of RD5B2) and used the same PCR cycle conditions as described above.

Amplification of ATPS fragment

A fragment of 235 bp of the nuclear ATPS was amplified using the sponge specific primers ATPSb_Ph_Fwd 5'-TGT CTT GGA AAA GGA AGG ATC AAA GG-3' and ATPSb_Ph_Rev: 5'-CGT TCA TTT GAC CAT ACA CCA GCG-3' (Bentlage and Wörheide, 2007) and the following cycling parameters: 3 min at 94°C, then 35 cycles of (94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s) and a final elongation of 10 min at 72 °C. To obtain the largest possible fragment of the flanking exons of the ATPS gene (Bentlage and Wörheide, 2007) and to facilitate sequence verification by BLAST search (Altschul et al., 1990), the degenerated exon primed intron crossing (EPIC) primers ATPSBF1/ATPSBR1 (Jarman et al., 2002) were used on eight random specimens. They yielded a bigger fragment of 295 bp containing 123 bp (73 bp at the 5' extremity and 50 bp at the 3' extremity) of the exon sequence, flanking the phase 0-intron that follows the GT-AT rule. PCR cycling conditions included 2 min at 94 °C, 35 cycles of 94 °C for 20 s, 55 °C for 60 s, and 72 °C for 50 s, and a final extension step at 72 °C for 10 min. These eight EPIC-PCR products were checked for their identity with the shorter fragments amplified with the sponge specific primers and their homology with sponge sequences was investigated using a BLAST similarity search (Altschul et al., 1990) with the sequences published by Wörheide et al. (2008).

Putative different nuclear intron copies within individuals require the ATPS marker to be treated cautiously, and to resolve the two alleles for analyses at the population-level. In this study, where the nuclear gene ATPS is used for taxonomic and phylogenetic purposes, chromatograms of both forward and reverse sequences were checked for length and sequence

variants. No length variation was observed within individuals and at most three positions out of 235 bp (representing a maximum ambiguity of 1.3%) showed double peaks in the chromatogram. These ambiguous positions were encoded using the IUPAC ambiguity code (Cornish-Bowden, 1985) and accounted for less variation than that observed within clades (1.3-6.3%).

PCR product processing and sequencing

PCR products were loaded onto a 1% agarose gel to check the size of the amplified product. The PCR products were then sequenced directly in both directions through a Perkin-Elmer ABI 3130 capillary DNA sequencer. PCR products were purified using exonuclease I, *Escherichia coli* (20 U μl^{-1}; Fermentas) and Calf Intestine Alkaline Phosphate (CIAP) (1 U μl^{-1}; Fermentas). Sequence files were read and assembled using DNASTAR Lasergene SeqMan Pro v.7.1.0. Because of the enhanced chance of amplifying symbionts or ingested DNA templates in encrusting sponges, sequences were verified for their poriferan origin by BLAST searches against the GenBank database (http://blast.ncbi.nlm.nih.gov/BLAST/) and with a cladistic tree-reconstruction as described in Erpenbeck et al. (2002).

All sequences are deposited in the NCBI GenBank nucleotide sequence database under Accession Nos. FN667709-FN667740.

Sequence alignment and phylogenetic analyses

Nucleotide data of COI, 28S, and ATPS fragments were used for phylogenetic analyses. Alignments were performed using the web interface of the multiple alignment software MAFFT (Katoh et al., 2002; available at http://www.ebi.ac.uk/Tools/mafft/index.html), using the default parameters. Sequences from *Aplysina fistularis* (Verongida, Aplysinidae, AY561987 and AY561864) and *Porphyria flintae* (Verongida, Aplysinellidae, Erpenbeck et al., unpublished data), were added as outgroup for the COI and the 28S datasets. Due to high evolutionary rates, ATPS intron sequences obtained from closely related Verongida species were unalignable. Therefore, midpoint rooting was used in the phylogenetic reconstruction of the ATPS fragment.

Phylogenetic reconstructions were performed under Maximum Likelihood (ML) and Bayesian inference (BI) criteria on each of the 28S, COI, and ATPS nucleotide datasets. BI analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under the best-fit evolutionary model estimated for each independent gene under the Akaike Information Criterion (AIC) with MrModeltest 1.1 (Nylander, 2002). The models selected by AIC were GTR + I + G for the COI partition, HKY + G for the ATPS partition and K80 + I for the 28S rDNA partition. Four Markov chains were run for 1 million generations and sampled every 1000 generations. The convergence diagnostic (the average standard deviation in split frequencies) was <0.01 at the end of the run. The trees of the first generations (250 trees) were discarded until the probabilities reached a stable plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree. Only posterior probabilities >0.95 were considered to support clades. Phylogenetic analyses under the ML criterion were conducted in PAUP* 4.0b10 (Swofford, 2002). The best-fit likelihood models (TVM + I + G for the COI partition, HKY + G for the ATPS partition and K80 + I for the 28S rDNA partition) were estimated with Modeltest 3.06 (Posada and Crandall, 1998) and selected using the AIC. ML trees were calculated using heuristic searches and a tree bisection and reconnection (TBR) branch swapping algorithm (10,000 rearrangements) with random stepwise addition of sequences in 100 replicate trials. Robustness of the ML trees was tested by bootstrapping with 100 replications and 10 replicate trials of sequence addition. Bootstrap values >70 were considered high enough to support clades in ML reconstructions.

Because a partition homogeneity test conducted using PAUP (Swofford, 2002) on COI and ATPS sequences (p = 0.11), on COI and 28S sequences (p = 0.25), on ATPS and 28S sequences (p = 0.68) and on the triple combination (i.e., ATPS+COI+28S; p = 0.08) indicated that data partitions were not significantly incongruent, gene partitions were combined for specimens from which all three sequences were available.

The combined dataset was analyzed using both Bayesian and Maximum likelihood inference criteria. Separate substitution models corresponding to data partitions were used for the concatenated data set in BI whereas GTR + G + I was used for a partitioned ML analysis using the program RAxML v7.2.5 (Stamatakis et al., 2006). In order to maximize the information content, a concatenated dataset with all sequences, including specimens showing missing data for a particular gene, was also analyzed.

Corrected p-distances (maximum intra-clade and minimum inter-clade) between clades for the COI and ATPS gene fragments were calculated in PAUP* 4.0b10 (Swofford, 2002) using the respective models from the ML analyses.

Results

mtDNA COI dataset

The resulting dataset comprised 46 sequences and 11 different haplotypes, with 657 nucleotides (81 variable, 45 of which parsimony informative). We observed seven non-silent mutations in the COI amino-acid dataset which all resulted from a change in the first codon-position. *Hexadella* species formed a well supported monophyletic group, with high Bayesian posterior probabilities and ML bootstrap support (1.0/100) (Fig. 2). *Hexadella* sp. (Seq1) was divergent from the other *Hexadella* specimens, which formed three highly supported clades (H1, H2, and H3). All specimens identified as *H. racovitzai* formed a monophyletic clade (clade H2), with representatives from the Mediterranean Sea (Marseille-Monaco, Banyuls), and the Atlantic (Gorringe, Rathlin Island in Northern Ireland, Plymouth in England) sharing a common haplotype. Two specimens from the Mediterranean Sea (Marseille-Monaco and the deep-sea sample from Port-Cros) differed in 16 bp positions from the common haplotype. Hereafter, the specimen from Port-Cros will be referred to as *H. racovitzai*. Clade H1 comprised specimens identified as *H. pruvoti* from Monaco and Marseille and *H. dedritifera* from the Ionian Sea, the Bay of Biscay, and from the Irish margin (D23ROC). The two haplotypes differed by only a single base mutation. The third well-supported clade (H3) corresponded to the deep-sea species *H. dedritifera* from the NEA, including samples from the Irish (Rockall Bank), the Scottish (Mingulay), and the Norwegian margin (Bergen, Røst reef) as well as from the Greenland Sea. Within clade H3 some substructuring was observed, with a highly supported Irish-Scottish-Norwegian subclade. Clearly, specimens identified as *H. dedritifera* belonged to two highly divergent clades (H1 and H3). Similarly, *H. pruvoti* specimens from the Gorringe Bank (P67gor, P73gor) located off the southwest coast of Portugal (hereafter Seq2) were clearly divergent from the *H. pruvoti* specimens occurring in the Mediterranean Sea (H1).

Minimum genetic divergence values between the three supported COI clades (H1-3) and the two divergent sequences (Seq1, 2) ranged from 3.9 % to 8.7 % (TVM corrected *p*-distance). Surprisingly, maximum genetic variation within H1 containing specimens from the shallow-water *H. pruvoti* and the deep-sea *H. dedritifera* was very low (0.4 %) while minimum genetic divergence between specimens morphologically identified as *H. dedritifera* (H1 and H3) was much higher (4.2 %). In addition, minimum genetic divergence between specimens identified as *H. pruvoti* (H1 vs. Seq2) was 3.9 % (Table 2).

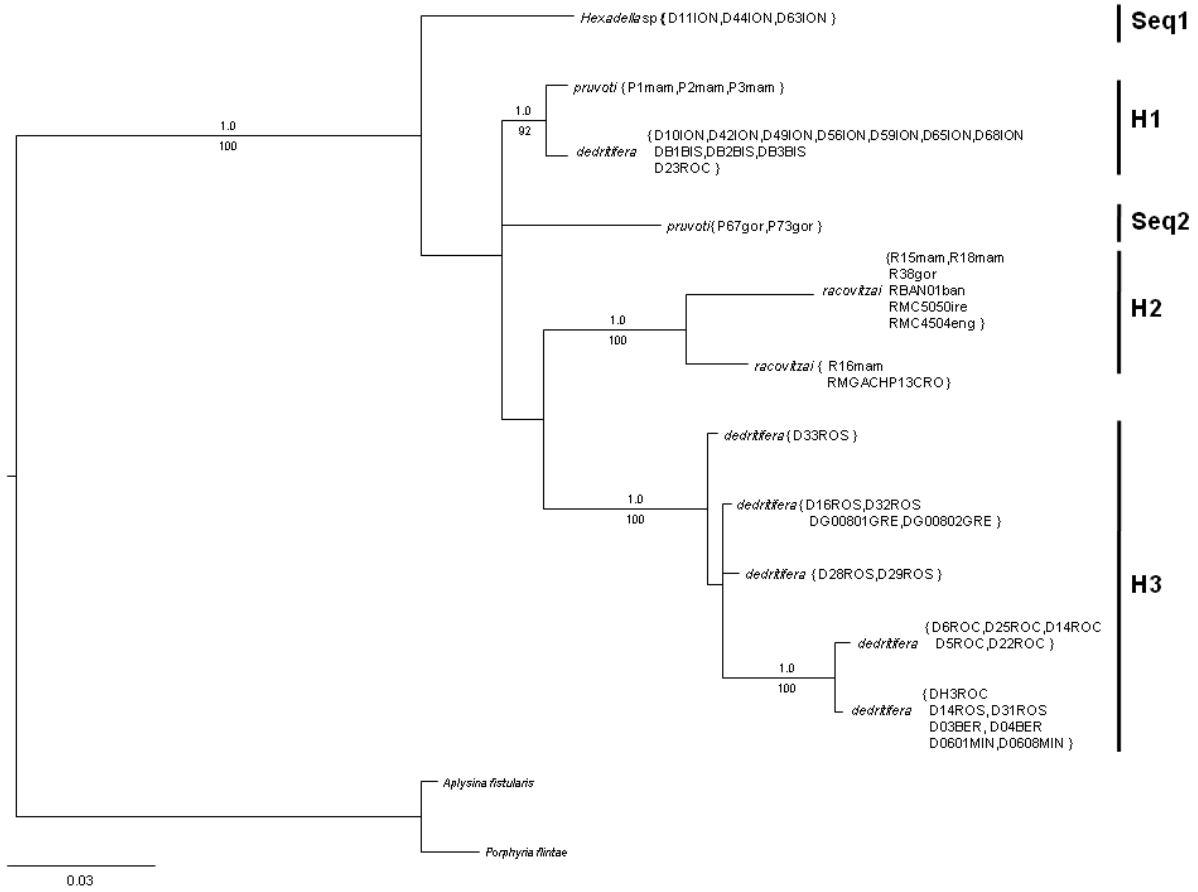

Fig 2. Bayesian majority-rule consensus tree of the mtDNA COI fragment. Bayesian posterior probabilities (when > 0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Table 2. Genetic divergence (corrected p-distance) between terminal clades (below diagonal) and between individuals within terminal clades (on diagonal) for mtDNA (COI) and nuclear (ATPS intron) markers. The different clades are presented in Figs. 2-5.

COI	**H3**	**H1**	**Seq2**	**Seq1**	**H2**
H3	0.023				
H1	0.042	0.004			
Seq2	0.062	0.039	0.000		
Seq1	0.071	0.042	0.070	0.000	
H2	0.064	0.058	0.062	0.087	0.035

ATPS	**H3**	**H1**	**Seq2**	**Seq1**	**H2**
H3	0.013				
H1	0,100	0.063			
Seq2	0.181	0.205	0.000		
Seq1	0.150	0.147	0.252	X	
H2	0.172	0.169	0.285	0.170	0.038

ATPS dataset

The resulting dataset comprised 33 sequences with 235 nucleotides (82 variable, 42 of which parsimony informative), collapsed into 11 different unambiguously alignable sequences. The three clades (H1-3), previously detected with COI, were also recovered with high Bayesian posterior probabilities and ML bootstrap support in the analysis of the ATPS intron (Fig. 3). The deep-sea *H. dedritifera* specimens from Clade H1 now formed a well supported subclade (0.99/96); the *H. pruvoti* specimens from the Marseille and Monaco regions differed by 15-19 bp from this subclade. H2 was shown as a subclade of a larger clade now including the Ionian Sea *Hexadella* sp. specimen (Seq1). This subclade (H2) contained again all *H. racovitzai* specimens, with the deep-sea *H. racovitzai* specimen from Port-Cros differing from the remaining specimens by 9 bp. Clade H3 contained two haplotypes that differed in 3 bp from each other. One haplotype was present in the deep-sea *H. dedritifera* specimens from the Irish (Rockall Bank), the Scottish (Mingulay), and the Norwegian

margins (Bergen, Røst reef), and the other haplotype was found in samples from Norway (Røst reef), and the Greenland Sea. The *H. pruvoti* specimens from the Gorringe Bank (Seq2) were again highly divergent from the *H. pruvoti* samples from the Mediterranean Sea (H1).

The minimum genetic divergence between the three supported ATPS clades (H1-3) and the two divergent sequences (Seq1, 2) were about twofold the values found with COI, and ranged from 10 % to 28.5 %. Maximum intra-clade variation within H1 was lower (6.3 %), supporting the lack of genetic divergence between the shallow-water *H. pruvoti* specimens (P1mam, P2mam, P3mam) and the deep-sea *H. dedritifera* specimens in the Ionian Sea, the Bay of Biscay and the Rockall Bank. Similarly, the low variation observed within H3 (maximum HKY corrected *p*-distance of 1.3 %) suggests a lack of genetic divergence between the Irish, the Scottish and the Norwegian margins. The high genetic divergence values between H1 and H3 (minimum HKY corrected *p*-distance of 10 %) and between H1 and Seq2 (minimum HKY corrected p-distance of 20.5 %) confirmed the occurrence of sharp genetic breaks within *H. dedritifera* and within *H. pruvoti*, respectively.

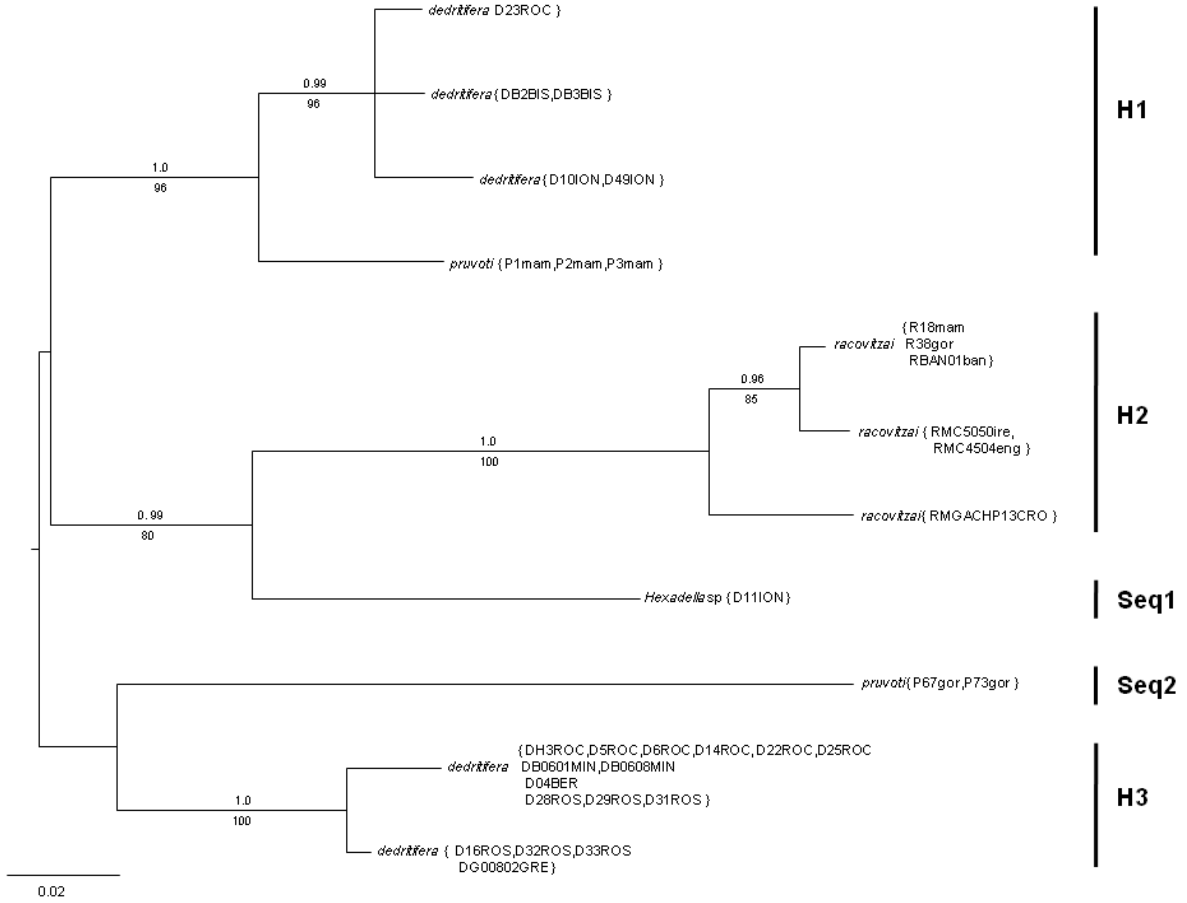

Fig 3. Bayesian majority-rule consensus tree of the nuclear ATPS intron. Bayesian posterior probabilities (when >0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. The tree is midpoint rooted. For information on the specimens see Table 1.

28S rDNA dataset

The resulting dataset comprised 33 sequences with 411 characters (9 variable, 3 of which were parsimony informative), collapsing into 10 different haplotypes. Phylogenetic resolution in the nuclear ribosomal 28S gene was clearly lower than in the two other markers, but species of the genus *Hexadella* were nevertheless corroborated as a monophyletic group (Fig. 4), with high Bayesian posterior probabilities and high ML bootstrap support (1.0/99). This marker was, however, unable to discriminate the different *Hexadella* species. For instance, *H. pruvoti* (mam) and *H. dedritifera* specimens from France (Bay of Biscay), Scotland (Mingulay), and Norway (Røst reef, Bergen) were shown as a single sequence.

Sequence divergence (K80 corrected *p*-distance) was about 10-fold smaller for 28S rDNA than for COI and 25-fold smaller than for ATPS (data not shown).

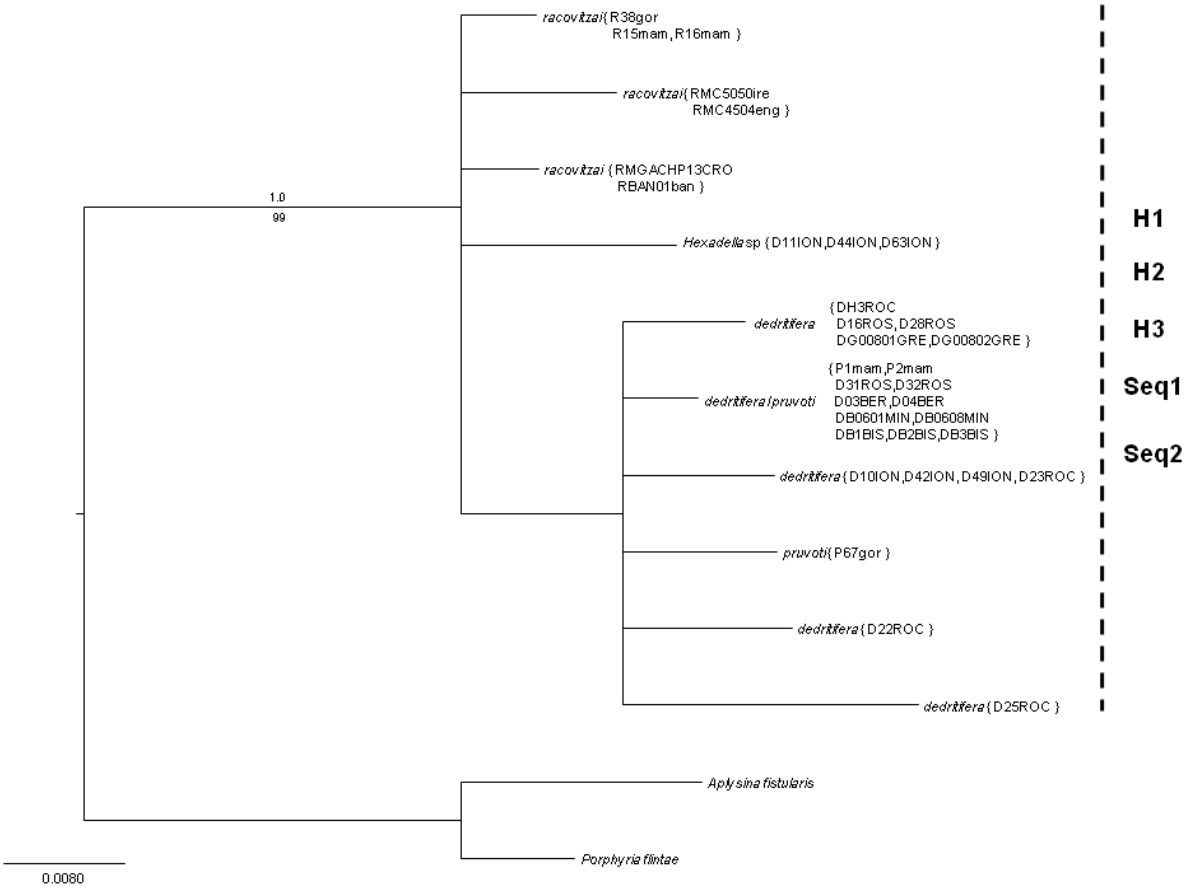

Fig 4. Bayesian majority-rule consensus tree of the rDNA 28S fragment. Bayesian posterior probabilities (when >0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Concatenated COI-ATPS-28S dataset

The resulting dataset comprised 25 sequences, with 1303 nucleotides. *Hexadella* spp. were confirmed as a monophyletic group, with high Bayesian posterior probabilities and high ML bootstrap support (1.0/100). The three clades (H1-3) and the two divergent sequences (Seq1, 2) were recovered by both analytical methods with higher support from the combined dataset than from the individual datasets of each marker. *Hexadella dedritifera* from clade H3 is corroborated in the concatenated dataset as highly divergent from *H. dedritifera* from clade H1. Similarly, Seq2 is corroborated in the combined dataset as clearly divergent from the *H. pruvoti* specimens occurring in the Mediterranean Sea (H1) (Fig. 5). Extending the concatenated dataset with specimens showing missing data for a particular gene resolved the deeper nodes more and Seq1 was placed basal to all other clades (data not shown).

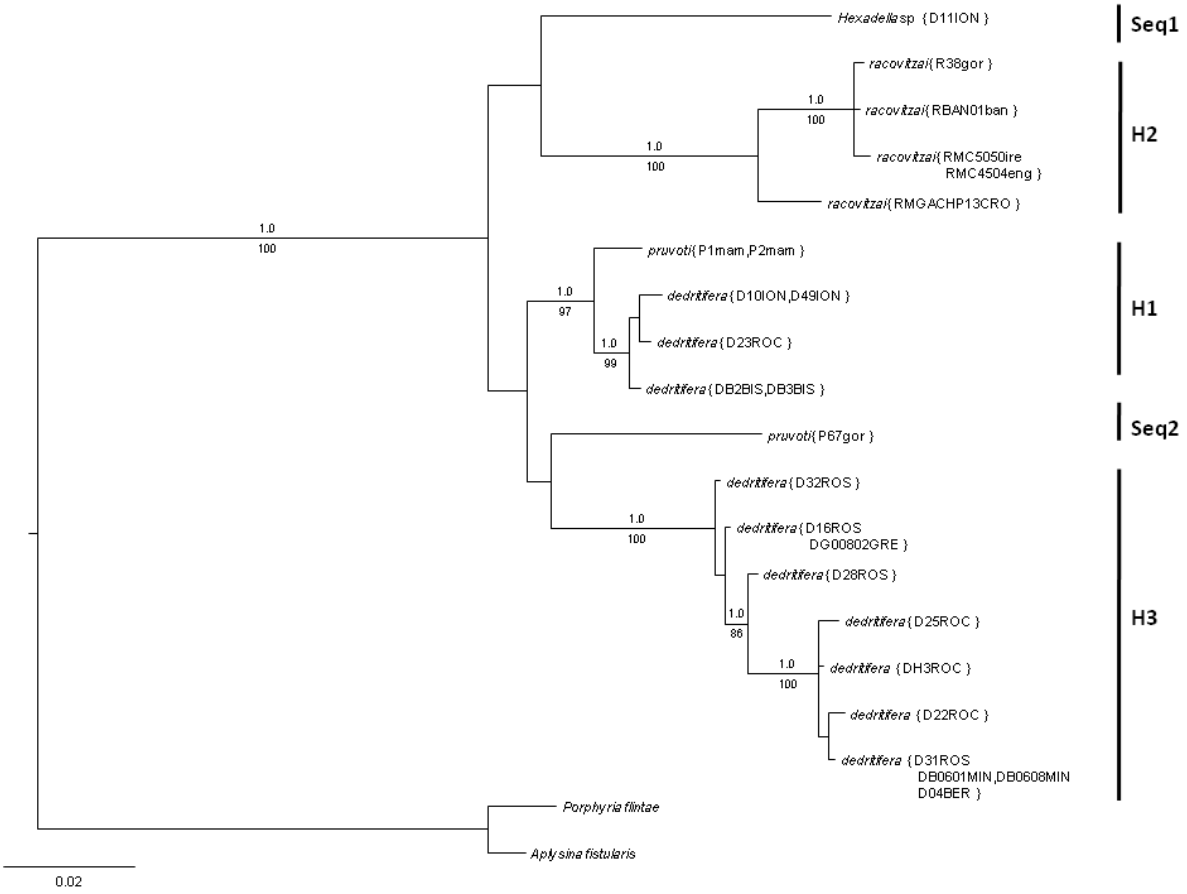

Fig 5. Bayesian majority-rule consensus tree of the concatenated dataset (COI-ATPS-28S). Bayesian posterior probabilities (when >0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens see Table 1.

Discussion

Our results demonstrate for the first time the presence of cryptic, deeply divergent lineages in a major group of benthic marine invertebrates associated with deep-water coral ecosystems along the European margin. In its current conception the deep-sea species *H. dedritifera* is polyphyletic, with two highly divergent clades H1 and H3 (minimum corrected *p*-distances of 4.2 % in COI and 10 % in ATPS). No COI threshold was defined to separate Evolutionary Significant Units (ESU sensu Moritz, 1994) because great differences in rates of evolution between sponge groups have been reported (Solé-Cava and Boury-Esnault, 1999; Cárdenas et al., 2007). Nevertheless, the observed genetic distances between H1 and H3 for ATPS are comparable with those found between putative sibling species of *Leucetta chagosensis* (Wörheide et al., 2008), suggesting that *H. dedritifera* may also be a species complex. The material from the Irish, the Scottish, the Scandinavian margins and the Greenland Sea in the NEA (Clade H3) probably represents *H. dedritifera sensu* Topsent, 1913 because of its origin close to Bear Island (Norway), the type locality. Following this, *H. dedritifera* specimens from clade H1 should be called *H.* cf. *dedritifera* until a proper redescription is made.

At the same time, the Mediterranean *H. pruvoti* and *H.* cf. *dedritifera*, both bright-yellow colored, form a strongly supported monophyletic group (H1). The low intra-clade variation within H1 in COI and in ATPS (maximum corrected *p*-distance of 0.4 % and 6.3 % respectively) stresses the lack of[14] genetic divergence between the shallow-water *H. pruvoti* specimens and the deep-sea *H.* cf. *dedritifera* specimens in the Mediterranean Sea. We may consider that one single species, *H. pruvoti*, occupies a wide bathymetric range in the Mediterranean Sea. However, morphological discriminating criteria such as choanocyte chamber size or incorporation of foreign material need to be reassessed, in our opinion, before synonymizing both species.

In addition, the results highlight the occurrence of significantly divergent lineages of specimens morphologically identified as *H. dedritifera* and isolated from the same cold-water coral ecosystem: D23ROC (clade H1) is clearly distinct from all other specimens from the Irish cold-water coral reefs (Rockall Bank, clade H3), indicating the existence of cryptic

[14] Surprisingly small instead

lineages of deep-sea sponges. Similarly, a putative new *Hexadella* species (Seq1) associated with the deep-sea reefs in the Ionian Sea was found to diverge from the *H.* cf. *dedritifera* (genetic distance of 4.2 % in the COI fragment and of 14.7 % in the ATPS fragment). This[15] suggests that the deep-sea coral ecosystems represent diversity hot-spots.

Despite their morphological similarity, the shallow-water individuals from the Atlantic Gorringe Bank (Seq2) were well differentiated from the Mediterranean Sea *H. pruvoti* (Clade H1), both in the COI and the ATPS (corrected *p*-distance around 3.9 % and 20.5 % respectively). The type locality of this species is Banyuls (France, Mediterranean Sea, Topsent, 1896), and therefore specimens from the Gorringe Bank most likely represent a new species, hereafter called *H.* cf. *pruvoti,* that will require proper taxonomic description.

In contrast to *H. dedritifera* and *H. pruvoti*, the specimens of *H. racovitzai* from the Mediterranean Sea (Marseille-Monaco, Banyuls, Port-Cros), the Atlantic Gorringe Bank and the United Kingdom (Rathlin Island in Northern Ireland, Plymouth in England) were genetically closely related (monophyletic). The deep-sea specimen from Port-Cros was the first *H. racovitzai* reported from the deep sea and was shown as a divergent sequence in both the ATPS and the combined dataset. Nevertheless, more individuals per population would be necessary to unravel the intraspecific diversity of this species, and to elucidate possible cryptic species patterns.

The mitochondrial cytochrome c oxidase subunit I (COI) gene and the D3-D5 region of the nuclear large ribosomal subunit (28S rDNA) have been repeatedly used in sponge systematics (Erpenbeck et al., 2002; Duran and Rützler, 2006; Erpenbeck et al., 2006a; Wulff, 2006; Blanquer and Uriz, 2007). In contrast, the second intron of the nuclear ATP-synthetase beta subunit gene has only recently been shown to provide a high resolution at the intraspecific level in sponge evolutionary studies (Bentlage and Wörheide, 2007; Wörheide et al., 2008). Although mitochondrial genes are maternally inherited and may follow different evolutionary pathways than their nuclear counterparts (Ballard and Whitlock, 2004), the topologies obtained with COI and ATPS were congruent. In addition, the ATPS marker showed a substitution rate more than twofold higher than the mitochondrial one. Our analysis

[15] These newly highlighted cryptic species add to the growing evidence that CWC reefs are diversity hotspots.

proved that the ATPS intron marker is useful for taxonomic purposes within the genus *Hexadella* and suggests its use may be of great interest as a complement for mitochondrial markers in sponge molecular studies. Nevertheless, two ATPS haplotypes instead of five in the COI dataset were shown in clade H3, possibly because a highly variable position of the ATPS partition showed some double peaks in the chromatogram and could not be resolved. It suggests that the ATPS marker might be of great potential for future phylogeographic studies within *Hexadella* species and for deeper insights into gene flow patterns, provided that the two alleles of the intron are retrieved. As expected, the 28S (D3-D5) gene fragment showed the lowest resolution (maximum pairwise genetic distance of 1.8 %), and was in general inappropriate to assess the phylogenetic relationships among the studied material. However, our 28S partition was only 411 bp long, and the use of a longer fragment might increase the resolution of the marker. For example, the (D1-D2) partition of the 28S gene evolves at a slightly higher rate than COI in Geodiidae sponges (Cárdenas et al., 2010) and might add resolution to the phylogeny of our *Hexadella* specimens. Despite high genetic distances, the deep phylogenetic nodes between H1, Seq2 and (H2 + H3) were not supported in the COI phylogeny. This low resolution might be the result of either insufficient sampling of taxa and/or nucleotides (i.e., soft polytomy). Given the vastness of ocean margins and the wide distribution of sponge habitats, the existence of other undiscovered species remains very likely.

Interestingly, no single polymorphic site was observed in the COI sequences between the H1 specimens from the Ionian Sea, the Bay of Biscay, and the D23ROC specimen (Rockall Bank), and only 3 % genetic divergence was observed in the ATPS dataset for those *H.* cf. *dedritifera.* These very low genetic divergence values across more than 5200 km (Ionian Sea-Rockall Bank) may represent the first genetic evidence suggesting the connection of deep-water coral associates from the Mediterranean Sea up to the Irish margin. This connection could be the result of sporadic gene flow between intermediate (unsampled) localities. Reveillaud et al. (2008) suggested the Biscay canyons and slope to presumably act as a semi-continuous habitat for larval dispersal of stony corals, while gene flow has been reported in sponges between adjacent populations in the Mediterranean and the Atlantic littoral (e.g., Duran et al. 2004). Furthermore, it has been recently shown in laboratory conditions (Maldonado, 2009) that larvae from the order Verongida (*Aplysina aerophoba*) can swim for 7 days before settling, indicating a great potential for dispersal in some aspiculate

demosponges. This pattern is also in accordance with the sporadic gene flow described for the coral species *Lophelia pertusa* between reefs along the similar NEA continental margin (Le Goff-Vitry et al., 2004; Le Goff-Vitry and Rogers, 2005).

Similarly, the well-supported clade H3 including *H. dedritifera* specimens from the Irish, the Scottish, the Norwegian margins and the Greenland Sea suggests the connection of the northern latitude populations. A single COI haplotype shared between the Irish, the Scottish and the Norwegian margin can raise some discussion about its significance, given that COI is known to evolve slowly in sponges (Duran et al., 2004). However, a single ATPS haplotype was shared between these geographically distant regions. Given that ATPS was shown to evolve more rapidly than COI (Bentlage and Wörheide, 2007; Wörheide et al., 2008, this study), and taking into account that one critical mutational step could not be resolved due to the absence of allele resolution in this study, our results suggest a putative gene flow between these northern reefs.

De Mol et al. (2005) suggested post-glacial intensification of currents coinciding with periods of increased Mediterranean Outflow Water (MOW) into the NEA as a pathway for Mediterranean deep-water corals to repopulate the high-latitude reefs. Paleoecological U/Th datings also suggest that post-glacial currents may have driven cold-water corals and associated fauna from southern refugia (e.g., the Mediterranean Sea, the region off NW Africa, and the mid-Atlantic ridge) towards northern latitudes (Schröder-Ritzrau et al., 2005, Roberts et al., 2006). At this point, it remains unclear whether a northward or a southward colonization event occurred in deep-sea sponge species since branches were poorly supported and the distribution of haplotypes in *H.* cf. *dedritifera* within clade H1 or in *H. dedritifera* within clade H3 did not show clear geographical patterns. Further sampling of *H.* cf. *dedritifera* and *H. dedritifera* and exploration of each ATPS allele will be needed to fully understand the putative gene flow within these deep-sea species.

Conclusion

This phylogenetic study illustrates the evolutionary distinctiveness of several lineages within the genus *Hexadella* in both shallow-water environments and deep-water coral systems. Before this study, three species of *Hexadella* were described from the Northeast Atlantic and Mediterranean Sea: *H. dedritifera*, *H. pruvoti*, and *H. racovitzai*. Our

phylogenetic analyses, based on the congruence of three independent nuclear and mitochondrial markers have suggested three potentially new cryptic species: *Hexadella* cf. *dedritifera* (maybe a junior synonym of *H. pruvoti*), *H.* cf. *pruvoti* (from the Gorringe Bank) and *Hexadella* sp. (at the moment only known from a deep-sea coral bank in the Ionian Sea). Now that they were revealed by molecular markers, an 'a posteriori' search of diagnostic phenotypic characters and description of these putative cryptic species is the next step for taxonomists (Blanquer and Uriz, 2008). Signatures of both genetic differentiation as well as gene flow across large areas in the deep-sea *H. dedritifera* and *H.* cf. *dedritifera* suggest a complex evolutionary history of deep-sea sponges. These results stress the need to protect multiple lineages of cold-water coral reefs. This important aspect in the conservation of deep-sea resources will ensure the maintenance of various sources of biodiversity.

Acknowledgments

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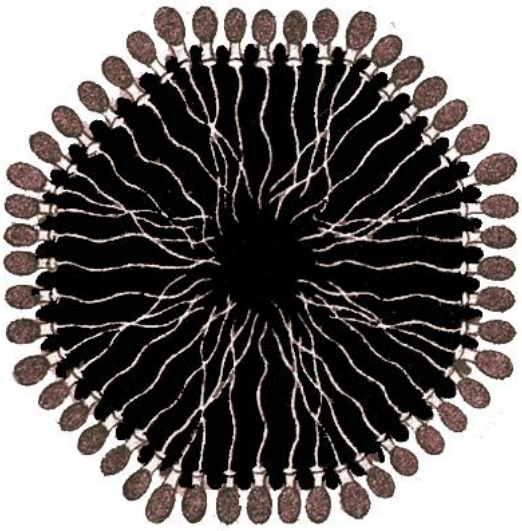
CHAPTER IV

Relevance of an Integrative Approach for Taxonomic Revision in Sponge Taxa: Case Study of the Shallow-water Atlanto-Mediterranean *Hexadella* (Porifera, Ianthellidae)



Manuscript in preparation

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Abstract

Background: The identification of sponges lacking mineral skeleton is always highly challenging, and this is especially the case for *Hexadella* species which are fiberless Verongida. Recently, a molecular analysis of European *Hexadella* species revealed highly divergent lineages within both shallow-water and deep-sea taxa. In shallow-water habitats, the yellow species *H. pruvoti* was identified as a complex of two cryptic species while the pink colored *H. racovitzai* showed two highly divergent lineages.

Methodology/Principal Findings: We performed a COI phylogenetic reconstruction using 27 new shallow-water *Hexadella* samples in order to confirm the presence of divergent lineages within both *H. pruvoti* and *H. racovitzai*. Specimens were retrieved from four shallow-water Mediterranean locations and described with an integrative approach combining morphological and cytological investigations, biochemical profiling and assessment of natural toxicity in order to identify diagnostic characters for each taxon. *H. topsenti* sp. nov. is distinguished from *H. racovitzai* by its color, the shape of its surface network, divergent secondary metabolite patterns and significantly different toxicity values. *H. crypta* sp. nov. differs from *H. pruvoti* by a different encrusting growth form when alive, and by distinctively coloring the ethanol when used as a fixative solution. The two species also harbor different types of cells with inclusions. Metabolic fingerprints, which are clearly different from *H. racovitzai*-like individuals, allow to separate *H. pruvoti* and *H. crypta*. Natural toxicity values however, do not permit distinguishing between the two species. We provide comprehensive species diagnoses for the shallow-water Atlanto-Mediterranean *Hexadella* including the two new species.

Conclusions/Significance: Our work shows that only the use of a combination of complementary tools can provide relevant descriptions for some problematic taxa, especially when the former (commonly used) taxonomical characters are lacking. In the case of the *Hexadell*a sponges, which lack fibers and spicules, our integrative approach allowed the description of two new species and provided taxonomical characters for the shallow- water Atlanto-Mediterranean species which make them more easily identifiable.

Keywords: Taxonomy, Phylogeny, Morphology, Cytology, Biochemistry, Toxicity, *Hexadella*

Introduction

The advent of molecular techniques into sponge systematics has provided efficient clues to discriminate cryptic species, shedding light on an outstanding high level of the phylum's biodiversity (see Xavier et al. 2010a for a review). These novel species should be formally described to be made readily accessible to both the scientific and environmental management communities (Blanquer and Uriz, 2008). However, complementary taxonomical descriptions ensuring the value of the new discovery remain scarce. Such taxonomic descriptions are particularly challenging in a group which harbors few and/or highly plastic phenotypic characters.

The sponges of the Verongida clade lack a mineral skeleton and their identification is therefore always highly challenging. Among the Verongida, four groups are distinguished, almost exclusively by the structure and composition of their spongin fibers (Bergquist and Cook, 2002). However among the Ianthellidae, the *Hexadella* species are fiberless. The quest for diagnostic morphological characters for unambiguous species identification in this group is hence always a challenge (Bergquist and Cook, 2002). Yellow and pink conspicuous *Hexadella* spp. are known from shallow to deep marine environments (Topsent, 1896, 1913; Morrow and Picton, 1996; Picton and Costello, 1998; Longo et al., 2005, Van Soest and Lavaleye, 2005, Van Soest et al., 2007a; Xavier and Van Soest, 2007; Reveillaud et al., 2010), and they are particularly numerous in the Mediterranean Sea (open water, caves and canyons, personal observation). Furthermore, verongid sponges produce complex brominated tyrosine-derived compounds, which show a wide range of biological activities (e.g., anti-HIV, but see Matsunaga et al., 2005). This group of sponges is hence of particular interest for the pharmaceutical industry and requires unambiguous species delineation (Wu et al., 1986; Morris and Andersen, 1989; Erwin and Thacker, 2007; Erpenbeck and Van Soest, 2007).

Phylogenetic analyses within the *Hexadella* (Verongida, Ianthellidae) from European shallow to deep environments using one mitochondrial (COI) and two nuclear gene fragments (28S rDNA and the second intron of the ATPS beta subunit gene) revealed deeply divergent lineages (Reveillaud et al., 2010). The shallow-water species *H. pruvoti* was shown to be paraphyletic. All *H. racovitzai* specimens collected from the Mediterranean Sea (shallow and deep) and the Atlantic formed a monophyletic group, but two highly divergent lineages were

also found separated by more than 16 bp out of 657 bp in COI, representing about 2.5% of uncorrected genetic distance.

With this work we present the first comprehensive integrative taxonomy study of *Hexadella pruvoti*- and *H. racovitzai*-like individuals. Our first aim was to significantly increase the number of samples in the Mediterranean Sea to further investigate the occurrence of cryptic species within shallow-water *Hexadella*. With these results at hand, our second aim was to revise the taxonomy of the shallow-water Atlanto-Mediterranean *Hexadella* spp. by investigating their general and ultra-morphology, chemistry and natural toxicity. General bioassays of the crude extract integrate the response of all the metabolites present in the organisms, providing a holistic view of the chemical ecology of the species (See Marti et al., 2003). The sponge morphology was studied from the general shape with consistent characters such as color, surface structures, and distribution of openings of the aquiferous system, up to the ultrastructure of choanocyte-chambers and mesohylar cells. Special attention was given to the cells with inclusions which were already highlighted in the original species descriptions by Topsent (1896). Our final aim was to provide complete species descriptions, and thereby make these species available to the scientific community.

Material and methods

Sampling and COI amplification

A total of 27 new individuals, including 14 *H. pruvoti* and 13 *H. racovitzai*-like individuals were photographed and collected by means of Scuba diving at four different sites in the Western Mediterranean, French coast : in the Marseille area (the Grotte à Corail, Tiboulen du Frioul, Impérial de Terre) and in Monaco (Tombant du Loews; see Fig1; Table 1). The Grotte à Corail sampling locality, close to the Maire Island (Marseille) was the same sampling locality as in Reveillaud et al. (2010), although in this earlier study it was erroneously referred to as the 3 PP cave.

After their collection, individuals were transferred immediately into 95% ethanol for the molecular and chemical analysis and in a mixture of glutaraldehyde 2.5%, 0.4 M cacodylate buffer and filtered sea water (1 vol.: 4 vol.: 5 vol.) for the cytological analysis. Amplification of the 5’ partition of the cytochrome c oxidase subunit I (COI), sequences alignment and phylogetic analyses were done as in Reveillaud et al. (2010) for all the new

Hexadella specimens. Sequences were checked for similarities with the haplotypes from the above study. NCBI GenBank accession numbers of the corresponding nucleotide sequence are provided in the species descriptions.

Cytology

Sponge material was maintained in the fixative for at least two hours after which it was rinsed with a 0.4M cacodylate buffer. All specimens were then post-fixed for two hours in a 2% osmium tetroxide solution in sea water and rinsed in filtered sea water and distilled water. Specimens were passed through acetic acid (5%) and then fluorhydric acid (5%), both during one hour, in order to remove foreign calcareous material or spicules included in the sponge tissue. Samples were then dehydrated through a series of graded ethanol and then placed in a sequence of propylene oxide, propylene oxide - araldite and araldite baths. The samples were embedded in Araldite (4 replicas for each sample), and polymerized over night at 60°C (see Boury-Esnault et al., 1995; Ivaniševic et al., 2010).

Semi-thin sections (2 µm) of the embedded samples were made with a microtome using a glass knife, and stained with toluidine blue for light electron microscopy. These sections provided information on the ultra structure of the sponge, and were also used to locate areas of interest prior to ultra-thin sectioning. Ultra-thin sections were performed with an ultramicrotome using a diamond knife, and then placed on copper grids. The sections were stained with 2% uranyl acetate and 0.3% lead citrate. Observations of cellular structures were performed with a Zeiss EM 912 transmission electron microscope (TEM).

Mesohyl cells were observed with a particular focus on the cells with inclusions, as their presence was emphasized in Topsent's original description of *Hexadella* species and in the review by Dendy (1905). Information regarding diversity of these cells was gathered from bibliography (e.g., Vacelet, 1967). In the studied material, cells with inclusions could be divided in three types: spherulous cells with large granular inclusions, each containing a large and dense spherule surrounded by a vesicular layer and an outer homogenous osmiophilic dense ring (type 1); spherulous cells with large inclusions containing microgranules (type 2); microgranular cells containing microgranules (type 3).

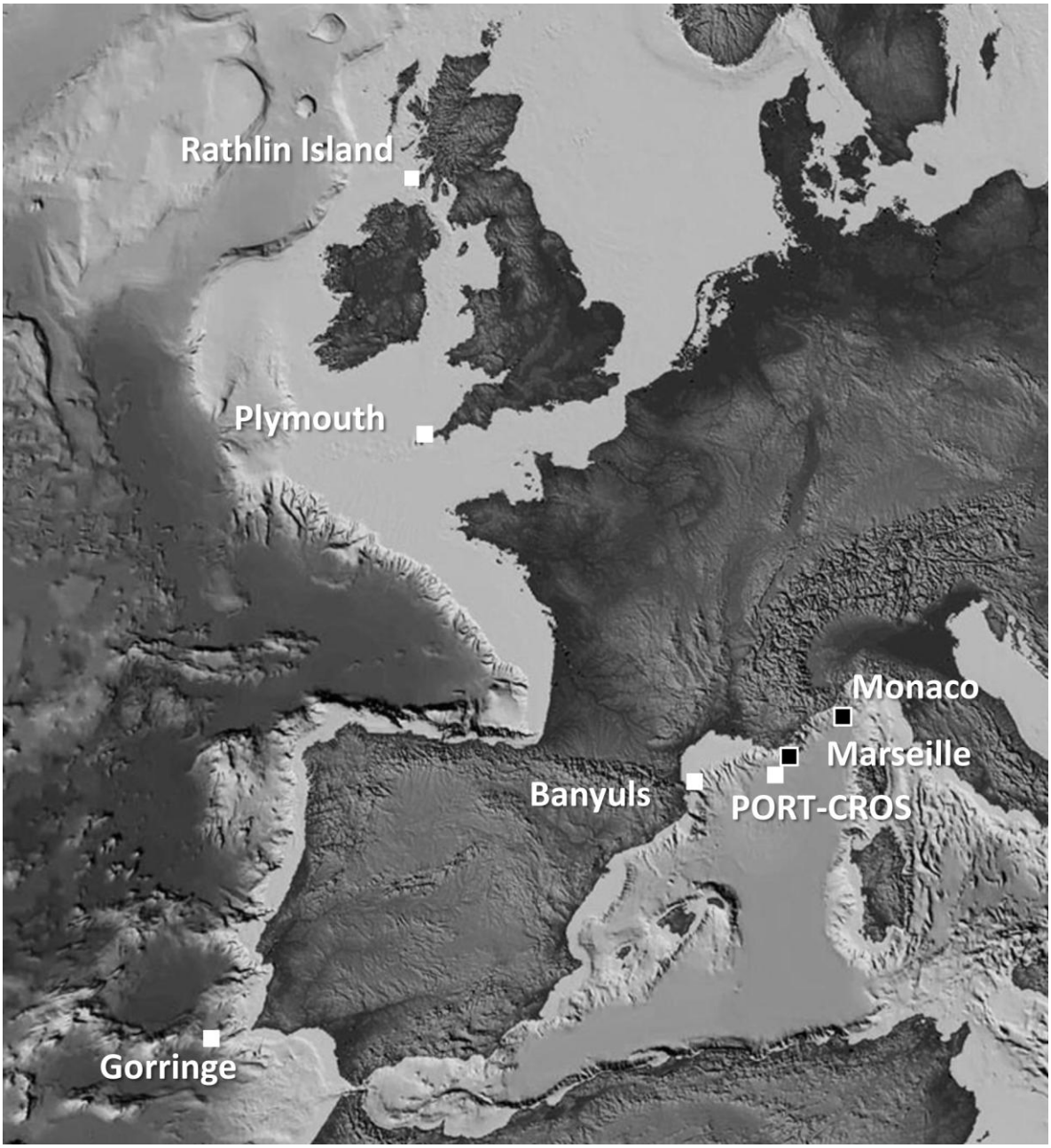

Figure 1: Map showing Atlanto-Mediterranean sampling locations of *Hexadella* species included in the COI phylogenetic analyses. Sampling locality is given in uppercase letters for deep-water sample (>50m; Port-Cros). Black squares refer to localities of new samples (this study) and white squares refer to sample localities from Reveillaud et al. (2010; included in the phylogenetic tree of the mtDNA COI gene).

Table 1: New *Hexadella* specimens analyzed in this study; information regarding their origin. Specimen number (No.), locality, coordinates, Maximum depth, external characters and identification.

No.	Locality	Coordinates	Max. Depth	Color/Characters	Species
Spe 1	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Bright-dark pink	*H. topsenti*
Spe 2	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Bright-dark pink	*H. topsenti*
Spe 3	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Bright yellow	*H. pruvoti*
Spe 4	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Faded-pale pink	*H. racovitzai*
Spe 5	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Faded-pale pink	*H. racovitzai*
Spe 6	Imperial de Terre, Marseille	43°10.37'N, 05°23.58'E	25	Bright yellow	*H. pruvoti*
Spe 7	Tombant du Loews, Monaco	43°44.15'N, 07°25.43'E	35	Faded-pale pink	*H. racovitzai*
Spe 8	Tombant du Loews, Monaco	43°44.15'N, 07°25.43'E	35	Faded-pale pink	*H. racovitzai*
Spe 9	Tombant du Loews, Monaco	43°44.15'N, 07°25.43'E	35	Faded-pale pink	*H. racovitzai*
Spe 10	Tombant du Loews, Monaco	43°44.15'N, 07°25.43'E	35	Faded-pale pink	*H. racovitzai*
Spe 11	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 12	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 13	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. pruvoti*
Spe 14	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright-dark pink	*H. topsenti*
Spe 15	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright-dark pink	*H. topsenti*
Spe 16	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright-dark pink	*H. topsenti*
Spe 17	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 18	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 19	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 20	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 21	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 22	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 23	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 24	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. crypta*
Spe 25	Grotte à Corail, Marseille	43°12.36'N, 05°19.57'E	10	Bright yellow	*H. pruvoti*
Spe 26	Tiboulen du Frioul, Marseille	43°16.815'N, 5°17.19'E	40	Faded-pale pink	*H. racovitzai*
Spe 27	Tiboulen du Frioul, Marseille	43°16.815'N, 5°17.19'E	40	Faded-pale pink	*H. racovitzai*

Data analysis and statistics

Most of the samples were examined with both thin and ultra-thin sections, except specimens 6,7,8,10,15 which were not observed through TEM (cf Table 1). Maximum length (µm) and width (µm) of every cell observed, were measured and further expressed as mean ± standard deviation (S.D) for each specimen studied. The measurements obtained were compared between distinct lineages and statistically processed with the STATISTICA software package (Statistica 7.1, StatSoft Inc., Tulsa, USA). All tests were carried out with

the non-parametric Kruskal-Wallis test followed by a two tailed test for multiple comparisons, since conditions for parametric testing were not fulfilled.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis of secondary metabolites was carried out on 15 specimens (Specimens number 1 to 15). 250 mg of sponge tissue were crushed in the ethanol used for preservation and filtered leading to the first extract. Sponge tissues were further extracted two times with a mixture (10 mL:10 mL) of methanol (MeOH) and methylene chloride (CH_2Cl_2) in an ultrasonic tank for 15 min each time. The combined filtered extracts were concentrated by vacuum rotary evaporation leaving a powdery organic residue. This residue was dissolved in MeOH/CH_2Cl_2 1:1, with a final concentration adjusted to 1 mg/mL, and was passed through a 13 mm, 0.2 µm PTFE syringe-filter before HPLC injection.

HPLC analyses were performed with a system from Waters including the Alliance separation module 2695, the column heater and the 2998 photodiode array detector. The equipment was controlled and the data were handled using the Empower Chromatography Data software (Waters). The HPLC elution conditions consisted of eluents A (0.1% aqueous trifluoroacetic acid) and B (0.1% trifluoroacetic acid in acetonitrile), an elution profile based on: an initial isochratic step at 30% B within 8 min, a linear gradient from 30% B to 80% B within 10 min, a linear gradient from 80% B to 100% B within 10 min and then 100% B for an additional 10 min. We used a Phenomenex Synergi Max-RP (80 Å, 250 x 3.0 mm, 4 mm) analytical column with a fixed temperature of 30°C and a flow rate of 0.4 ml. min^{-1}. 10 µl of the filtered solution was injected into the HPLC system.

In the present study, secondary metabolites profiling was restricted to the comparison of chromatograms extracted at 245 nm from the data collected across the 200 to 350 nm wavelength range. Chromatograms extracted at 245 nm indeed provided the best signal/noise ratio. The obtained chromatograms enable the detection of all detectable UV245-absorbing components in the samples. Retention times and UV spectra, extracted from the collected data, were used to compare peaks detected in the different specimens of a species or to compare peaks between the species. To maximize sensitivity, the data were also processed to create a Max Plot chromatogram which plots the maximum spectral absorbance measured at each time point. The Max Plot enables the detection of all detectable UV-absorbing

components in the sample. Isolation and characterization of the specimen's components was beyond the scope of the study.

Natural toxicity quantification

Natural toxicity quantification was carried out on all specimens except specimens 9,16-18, 22 and 26-27 (cf Table 1) using the standard Microtox® bioassay (Microbics, Carlsbad, CA, USA; Ribo and Kayser, 1987). Although this standard toxicity test has no known ecological meaning in itself, its results correlate well with ecologically relevant analyses (i.e., it integrates the response of all the metabolites present in the organisms) and it was found to be a reliable test for comparative bioassay studies in marine species (Becerro et al., 1995; Marti et al., 2003). The method analyses the sponge extract effect on the bioluminescence of the deep-sea bacteria *Vibrio fischeri*. Crude organic extracts (i.e., the powdery organic residue prepared for HPLC analysis) were dissolved in artificial seawater and up to 2 % of acetone was added to improve dissolution of the extracts. The solution was tested in four diluted concentrations. The initial concentration was set at 1000 µg/mL and a dilution factor of 2 was applied between each following concentration. The bioactivity was quantified by measuring the decrease in light emitted by the bacteria and expressed as an EC50 value. The lower the EC50, the more toxic the analyzed specimen is.

Results

mtDNA COI phylogenetic reconstructions

Among the 73 sequences included in our dataset, 46 came from a previous study by Reveillaud et al. (2010). The 27 new sequences showed the same four COI haplotypes as the former study (See Figure 2). This analysis supports the occurrence of significantly divergent COI lineages within both *H. racovitzai* (clade H2) and *H. pruvoti* (clade H1 vs. Seq2). The two *H. racovitzai* COI haplotypes, separated by a genetic divergence value comparable with those found between cryptic species within *H. pruvoti* (uncorrected p-distance of 2.5% and 3.3% respectively), were now recovered from 21 samples (8 from Reveillaud et al, 2010 and 13 in this study). These divergent lineages, also recovered in the nuclear marker ATPS (Reveillaud et al., 2010), hence confirmed the occurrence of the two clearly differentiated groups of *H. racovitzai* specimens, at different time points.

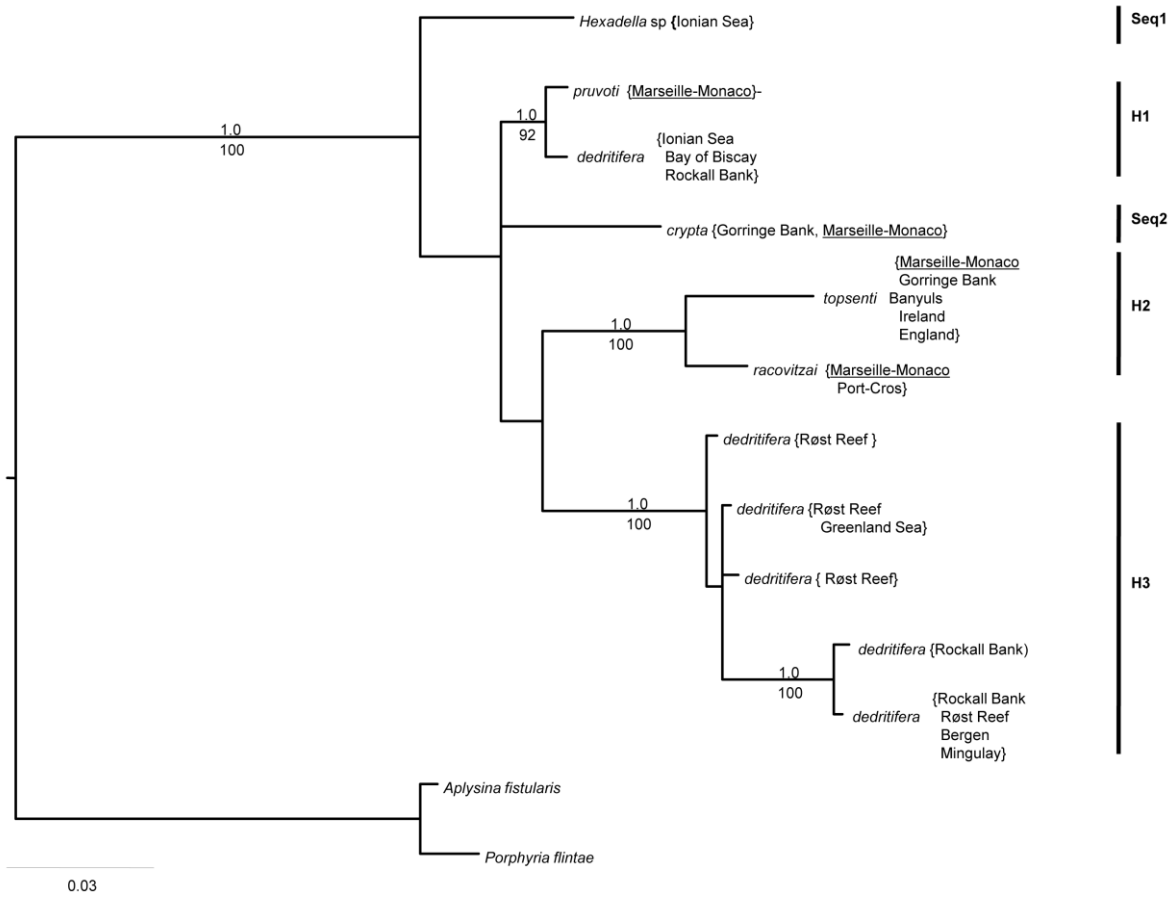

Fig 2. Bayesian majority-rule consensus tree of the mtDNA COI fragment. Bayesian posterior probabilities (when > 0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. New sample localities (this study) are underlined (Modified after Reveillaud et al., 2010; for more information on the localities see the former study).

Taxonomic descriptions

Family **IANTHELLIDAE**

Genus ***Hexadella*** Topsent (1896)

Type species: *Hexadella racovitzai* Topsent, 1896 (by original designation)

Diagnosis

Soft, thin encrusting Ianthellidae which lack a fibrous skeleton (Bergquist and Cook, 2002). General consistency is soft, skin-like and easy to tear. Surface is highly marked with tiny reticulated wrinkles, forming small conules when supported by underlying foreign material. Ostia are microscopic. The tough ectosome is strongly collagen reinforced, bounded by a distinct skin or cuticle (1µm), acting as external skeleton for the sponge mass and allowing the sponge to attain a thickness up to 5mm (Bergquist and Cook, 2002). Otherwise the body is unsupported, with many channels and lacunae. The choanosome is indeed fleshy and consists of large, sac-shaped choanocyte chambers (Dendy, 1905).

Remarks

Hexadella Topsent (1896) was transferred from Darwinellidae to Ianthellidae after histological studies, chemical analysis of secondary metabolites (Morris and Andersen, 1989) and DNA analysis. Electron microscopy confirmed that spherulous cells of Ianthellid type occurred in *Hexadella* (Bergquist and Cook, 2002). Affiliation of the Ianthellidae with the Verongida was also based on ultrastructure, secondary metabolite chemistry and reproductive behaviour (Bergquist and Cook, 2002). Of the eight species of *Hexadella* so far described, only six of them are valid (World Porifera Database, Van Soest et al., 2011): *Hexadella pruvoti* Topsent, 1896, *Hexadella racovitzai* Topsent, 1896 and *Hexadella dedritifera* Topsent, 1913, are all from the Atlanto-Mediterranean waters. The latter is a massive encrusting sponge, incorporating various particles of foreign debris in its tissue (Topsent, 1913). *Hexadella indica* Dendy, 1905 has been described from India as a possibly red sponge when alive, with a lamellate and folded choanosome similar to that of *Oscarella*, but with an ectosome which is rather similar to that of *Hexadella*. Its assignment to *Hexadella* rather than to *Oscarella* remains uncertain. According to Bergquist and Cook, 2002, *Hexadella kirkpatricki* (Burton, 1926) from South Africa, with stipate form and thick cortex is not considered as a *Hexadella* while the same authors consider *Hexadella purpurea* (Burton, 1937) from India is a synonym of *H. racovitzai*. *Hexadella pleochromata* (De Laubenfels,

1950) is now a synonym of *Pseudoceratina purpurea* (Bergquist and Cook, 2002; Van Soest et al., 2011). *Hexadella detritifera* Topsent, 1913 has been accepted as *Hexadella dedritifera* Topsent, 1913 (Van Soest et al., 2011).

Hexadella pruvoti (Topsent, 1896)

(Figure 4)

Original description.

Shallow water, Mélobésiées du Cap d'Abeille Banyuls, France, Western Mediterranean.

Material examined

Paratype n° MNHN xx. Specimen 3. Impérial de Terre at 25 m depth, Marseille, France

Other material examined

Marseille: one specimen (Specimen 6) from Impérial de Terre (25m), two specimens (Specimen 13, 25) from the Grotte à Corail (10m).

Genbank acc.number:FN667709 (corresponding to haplotype P3mam, see Reveillaud et al., 2010).

Diagnosis

Bright yellow *Hexadella in vivo*, turning dark purple in ethanol. Gives a yellow tint to ethanol. Body is large, lobate and thinly encrusting. Contains particular spherulous cells of type 1 with large inclusions of heterogeneous size, spherulous cells with large inclusions containing microgranules (type 2) and microgranular cells (type 3). Small choanocyte chambers (± 40 x 20 µm). Shows aerophobins 1 and 2 compounds. Medium-high natural toxicity.

Description of MNHN xx.

Thinly encrusting sponge with lobate body, bright yellow *in vivo*. It turns dark purple in alcohol whereas the fluid takes a yellowish color. Surface appears entirely striated and with microscopic pores (i.e., ostia) surrounding tiny conules (i.e., surface protrusion) showing some underlying debris. Large oscules are observed as elevations of the ectosome on living specimens (Fig. 3A). They are not visible after fixation in ethanol.

General organization

The ectosome is distinct from the fragile choanosome, and some ectosomal bundles of collagen fibrils are observed. Large packages of cells with inclusions and bacteria (Fig. 4A) are present in the mesohyl. The choanosome contains densely packed, large eurypylous choanocyte chambers of about (40 x 20) µm which contain an average of 40-60 choanocytes (Fig. 4B).

Cytology

Choanocytes are spherical to subspherical (4.3 ± 0.6) x (3.5 ± 0.7) µm in size, with central nucleolated nucleus of (1.9 ± 0.2) x (1.7 ± 0.2) µm. They are laterally bounded to one another and anchored in the mesohyl through short basal pseudopodia (Fig. 4B). A wide cytoplasmic collar, composed of microvilli surrounds the choanocyte. Pinacocytes are flattened, approximately (5.5 x 2.8) µm large, with long pseudopodia at both ends, and a large nucleolated nucleus occupying almost the entire cell. Three different types of cells with inclusions are distinguishable within the mesohyl. **The type 1** is represented by **spherulous cells with large granular inclusions** (Fig. 4D and F), abundant through the mesohyl and often regrouped. These cells are more or less spherical, slightly larger than the other types [see below: (11.2 ± 4.7) x (7.7 ± 3.1)] µm in size and contain about 40 to 50 inclusions of unequal size and shape. These inclusions occupy a large proportion of the cell content. Each inclusion contains a large and dense spherule surrounded by a vesicular layer, and an outer homogenous osmiophilic dense ring. The cell cytoplasm appears empty and its membrane is very thin (Fig. 4F). **The type 2** is represented by **spherulous cells with large inclusions containing microgranules**, observed in the mesohyl (Fig. 4C). These cells are about (8.9 ± 2,3) x (5.4 ± 1.4) µm in size and contain an anucleolate nucleus. The nucleus is compressed by the accumulation of 10 to 15 large inclusions occupying the entire cell, and therefore not visible in some sections. Each inclusion contains a high density of tiny osmiophilic electron dense granules. Some lateral expansions of the cytoplasm may be present (Fig. 4C). Intermediary developmental stages of these cells can be observed, from early stage to degeneration with rupture of their membrane and release of the granules in the surrounding mesohyl. The **type 3** is represented by **microgranular cells**, rare, (Fig. 4E) in the mesohyl. They are irregular shaped cells of about (7.3 ± 0.9) x (4.6 ± 1) µm in size, with a small nucleus and contain oval to rod-like osmiophilic electron dense granules (10-30 per cell).

Chemistry

We obtained HPLC traces with well observable (0.01 – 0.5 AU) and resolved peaks corresponding to the chemical fingerprints of each crude extracts of *Hexadella pruvoti* individuals (Specimen 3, 6 and 13). The three chemical fingerprints formed a homogeneous group in which all specimens showed in common: three intense (4.1, 6.4 and 8.1 min) and five minor (3.7, 5.5, 7.5, 9.9 and 11.7 min) peaks in the polar compounds zone (3-12 min); and an intense (16.5 min) flanked by two less intense (16.2 and 17.2 min) peaks, as well as a minor peak (around 20 min) in the medium polar compounds zone (15-22 min). The chromatogram of the Spe 3 extract (C, Fig.5) showed the most intense peak and was chosen as the representative chemical fingerprint for the species. Comparison of the UV spectra of the detected compounds from the three individuals clearly confirmed the homogeneity of this group. The UV spectra of the compounds eluted at 6.4 and 8.1 min were similar to those of aerophobins 1 and 2, bromotyrosine alkaloids that we isolated in *Aplysina aerophoba* (personal database). Specimen 6 was distinguishable from the two others by the presence of an additional peak at 18.6 min. The same observations could be made by comparison of Max Plot chromatograms (data not shown).

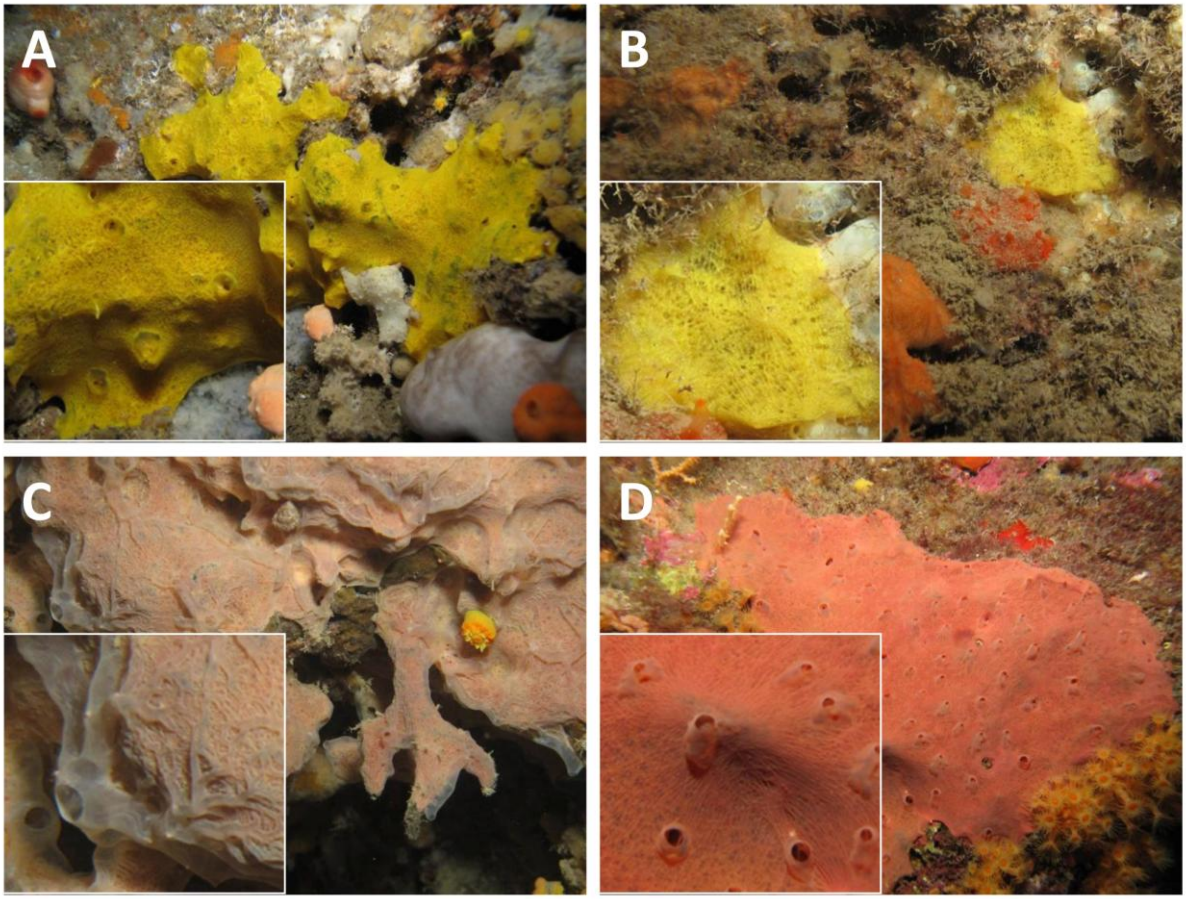

Figure 3- *In vivo* pictures of specimens of *Hexadella* species before their collection (photos by T. Pérez). A- Bright yellow *H. pruvoti*. Thinly encrusting on rocky substrate, body large, diffuse and lobate. Zoom-in: the highly wrinkled body of the organism, the micropores and the exhalant canals forming small chimneys leading to the oscula. B- Bright yellow *H. crypta* on rocky substrate, surrounded by other invertebrates. Zoom-in: The sponge appears as a small, poorly diffuse, cushion like encrustation entirely striated, with tiny conules on its surface. C- Thinly encrustation faded, pale pink *H. racovitzai* on rocky substrate, surrounded by other invertebrates. Zoom-in: the surface of the sponge, highly striated, with its conspicuous star-shaped network of canals, leading to wide openings, the oscula. D- Bright to dark pink *H. topsenti*. Thinly encrusting with lobate body, on rocky substrate. Zoom-in: the wrinkled body surface of the organism with its membrane-like "chimneys" leading to the oscula.

Natural toxicity quantification

Hexadella pruvoti crude extracts (3, 6, 13, 25) showed EC50 values ranging between 49 and 270 µg/ml. These values correspond to a medium-high natural toxicity.

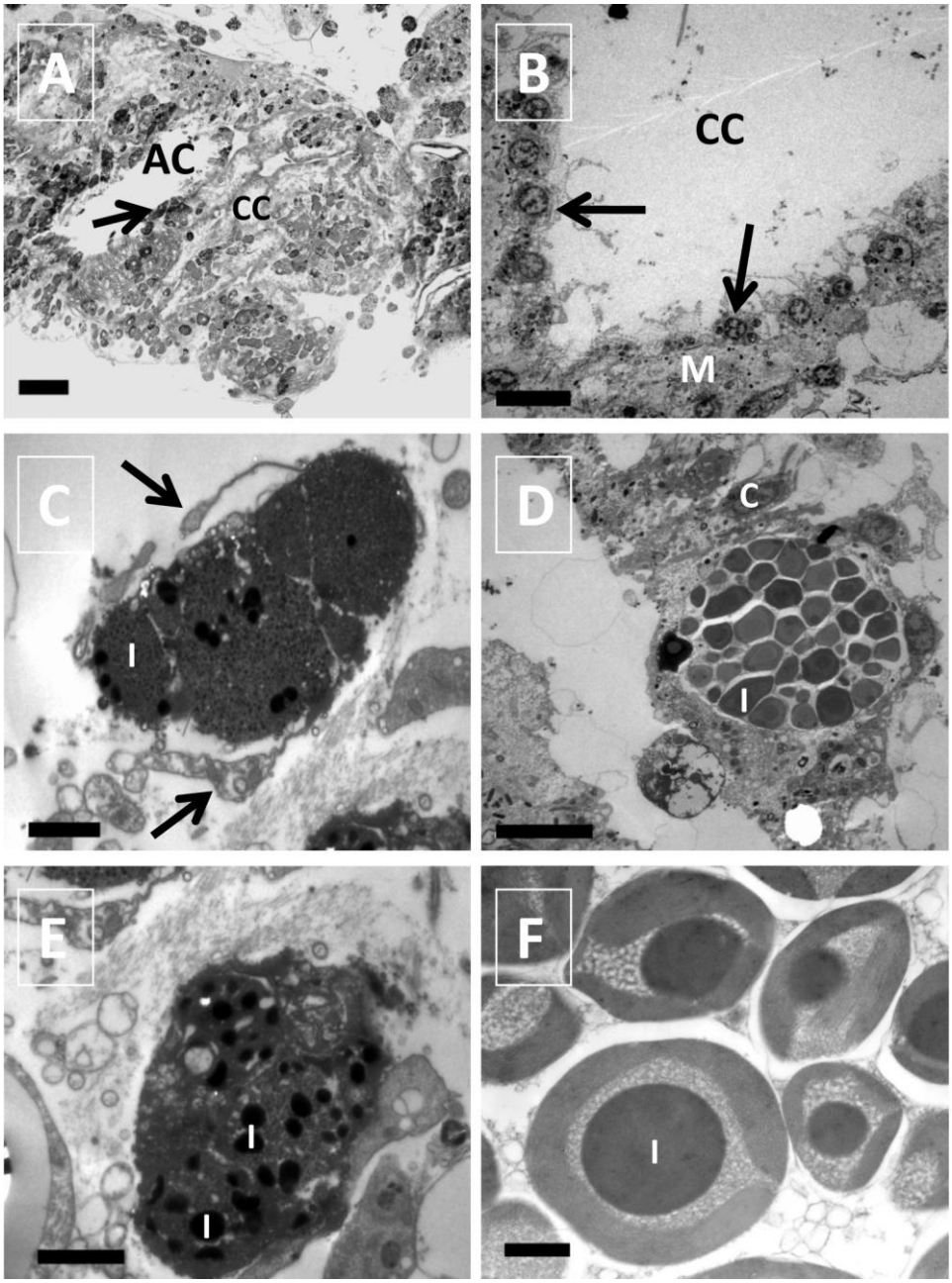

Figure 4: A, Semi-thin section through *H. pruvoti*. B-F TEM views of *H. pruvoti*. A- Represents the choanosome with its aquiferous canals (*AC*) surrounded by large eurypylous choanocyte chambers (CC). The choanosome contains a large amount of cells with inclusions often packaged (arrows), distributed throughout the mesohyl, within some choanocyte chambers and mainly concentrated around the aquiferous canals. B- Represents a choanocyte chamber (*CC*), composed of laterally attached choanocytes (arrows). The chamber appears empty compared to the surrounding mesohyl (*M*) which contains some bacteria. C- Spherulous cell type 2, containing large inclusions (*I*) with granular content. Notice the lateral expansions of the cytoplasm (arrows). D- Spherulous cell type 1, with heterogeneous sized inclusions (*I*) typical for *H. pruvoti*, passing through a choanocyte chamber, as indicated by the presence of choanocytes (*C*). E- Microgranular cell (type 3), containing small rod-shaped/spherulous inclusions (*I*) which are osmiophile and electron dense. F-Detail of an inclusion (I) from a spherulous cell type 1 .Scale bars: A=25µm B= 5µm, C=2µm, D=5µm, E=2µm, F=0.5 µm

Locality and habitat

The material was collected from Impérial de Terre, Mediterranean Marseille, France, on a coralligenous cliff at 25m. The species is widely distributed in the Western Mediterranean, but so far confirmed records are only from Impérial de Terre (25m), Grotte à Corail (10m), Marseille, France and Tombant du Loews (35m), Monaco (Reveillaud et al., 2010).

Taxonomic remarks

The description given by Topsent of this species was very brief and unfortunately without illustrations.

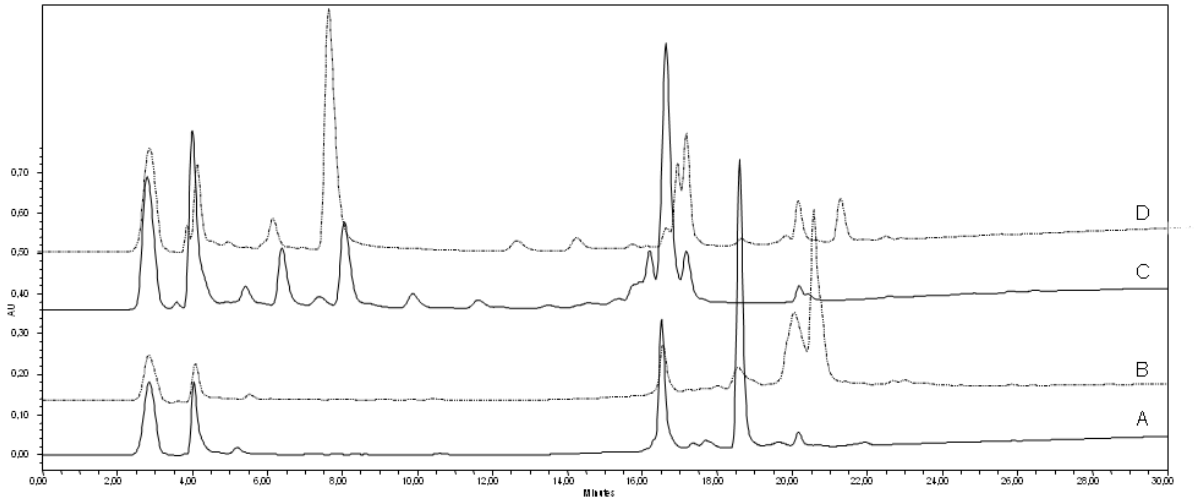

Figure 5: A-D Representative high-performance liquid chromatography chromatograms (UV 245 nm) of organic extracts of *H. racovitzai* specimens (A), *H. topsenti* (B), *H. pruvoti* (C) and *H. crypta* specimens (D). Chromatograms at 245 nm are extracted from data collected across the 200-350 nm wavelength range. Absorption unit (AU) is plotted as a function of time (minutes).

Hexadella crypta sp. nov.

(Figure 6)

Restricted synonymy. *Hexadella pruvoti*.

Material examined

Holotype. MNHN xx Specimen 11, Grotte à Corail, Mediterranean near Marseille, France (depth 10m)

Paratype. MNHN xx, specimen 12, Grotte à Corail (Marseille, France).

Other material examined

Marseille: eight specimens (Specimen 17-24) from the Grotte à Corail (10m).

Genbank acc.number: FN667712. (corresponding to haplotype P73gor, see Reveillaud et al., 2010).

Diagnosis

Bright yellow *Hexadella in vivo*, turning dark purple in ethanol. Gives a purple tint to ethanol. Small, cushion like encrustation. Contains spherulous cells with large inclusions containing microgranules (type 2) and microgranular cells (type 3). Small choanocyte chambers (± 30 x 20) µm. Shows aerophobins 1, 2 and isofistularin compounds. Medium to high natural toxicity.

Description of MNHN xx

The sponge consists of a small, cushion like, encrustation. Its body is slightly thicker than *H. pruvoti* and does not form lobes. The surface is entirely striated, and ostia are microscopic. Some pointed conules are visible on the sponge surface (Fig. 3B). Oscules are rare. Color of living specimens is bright yellow, although it comes in paler shades of yellow as well. The typical reaction of Verongids, turning purple when placed in alcohol is valid for this species. Noteworthy, the ethanol changes to the same purple color as the species itself.

General organisation

The ectosome is rigid, collagen fibrils are present, and it is non-detachable from the lacunar choanosome. Large packages of cells with inclusions (Fig. 6A, arrows) are present in the mesohyl as well as bacteria (one same type). Within the choanosome, large, eurypylous sac-shaped, choanocyte chambers of approximately (30 x 20) µm contain the choanocytes (Fig. 6A).

Cytology

Choanocytes (Fig. 6B) are spherical to subspherical, (3.9 ± 0.7) x (3.1 ± 0.6) µm in size, laterally bounded to one another. These are anchored in the mesohyl through short basal pseudopodia. Their nucleus is large, (2.7 ± 0.3) x (2.1 ± 0.4) µm, nucleolated (0.8 ± 0.3) x (0.7 ± 0.2) µm, and it occupies the center of the cell. A wide cytoplasmic collar, (Fig. 6C, arrow) surrounds the body of the choanocyte. Pinacocytes (Fig. 6D) are (8.5 x 3.2) µm large,

with long pseudopodia at both ends and a large nucleolated nucleus occupying almost the entire cell diameter. Two types of cells with inclusions are present within the mesohyl. Both cell types are shared with *H. pruvoti*. **Spherulous cells with large inclusions containing microgranules** (inclusion cells type 2) are found very abundant in the mesohyl (Fig. 6D-E). They are spherical to ovoid cells, (8.9 ± 2.3) x (6.7 ± 1.7) µm in size, with anucleolate nucleus. The nucleus is compressed by the accumulation of large inclusions and therefore not visible in some sections. Each inclusion contains a large amount of osmiophilic electron dense microgranules. Some lateral expansions of the cytoplasm may be present. Intermediary stages of development are frequently observed. Abundant **microgranular cells** (inclusion cells type 3; Fig. 6F) are distributed within the mesohyl. These cells are irregular in shape, (6.2 ± 2.1) x (3.8 ± 1) µm in size, and they contain a small nucleus, and rod-shaped highly osmiophilic granules.

Chemistry

Hexadella crypta specimens (Specimen 11-12) showed a chromatogram with well observable (0.01 – 0.6 AU) and resolved peaks (Fig.5, group D). Although the chromatograms of both specimen 11 and 12 were almost identical, the chromatogram of specimen 11 didn't show a peak at 17.3 min (see below) and thefeore the chromatogram of the Spe 12 extract is shown in Fig. 5 (D). This chemical profile was similar as in *H. pruvoti* if we compare the polar compounds' zone (3-10 min). Retention times and UV spectra of intense peaks (4.1, 6.0 and 7.4 min) were the same in both species. In the medium polar compounds' zone (12-22 min), *H. crypta* was characterised by the presence of two unseparated compounds (17 and 17.3 min) and a series of peaks (12.7, 14.3, 16.8, 18.7, 20.2 and 21.4 min). The UV spectra of these compounds between 12 and 22 min were superimposable and closely related to the one of isofistularin, a bromotyrosine alkaloid that we isolated in *Aplysina aerophoba* (personal database).

Toxicity Quantification

The EC50 values found for the *H. crypta* crude extracts (11, 19-21, 23-24) were about the same order as the ones found for *H. pruvoti*. They ranged between 49 and 270 µg/ml and thus revealed a medium to high natural toxicity.

Habitat and distribution

The material examined here was collected from the semi-dark cave of Grotte à Corail in Marseille, France, NW Mediterranean, at a depth of 10 m. The sponge has also some confirmed records from south Portugal, NE Atlantic (Gorringe bank, depth 39-42m; Reveillaud et al., 2010).

Taxonomic remarks

This species is difficult to distinguish from *H. pruvoti* but smaller in size, thicker, less encrusting, and less lobate. It can also be recognized through the fact that the alcohol itself undergoes the typical yellow to purple color reaction of Verongids. This species may have been misidentified by some authors because of its cryptic nature, and probably by the fact that both species are found side by side in the same location (e.g., Grotte à Corail, Marseille). In 2007, Xavier and Van Soest reported the first record of the species *H. pruvoti* in the Atlantic, but this could actually correspond to *H. crypta*.

Etymology

The name *H. crypta* recalls that this species was highly cryptic to taxonomists, and required a close and comparative analysis with congeneric taxa for its discrimination.

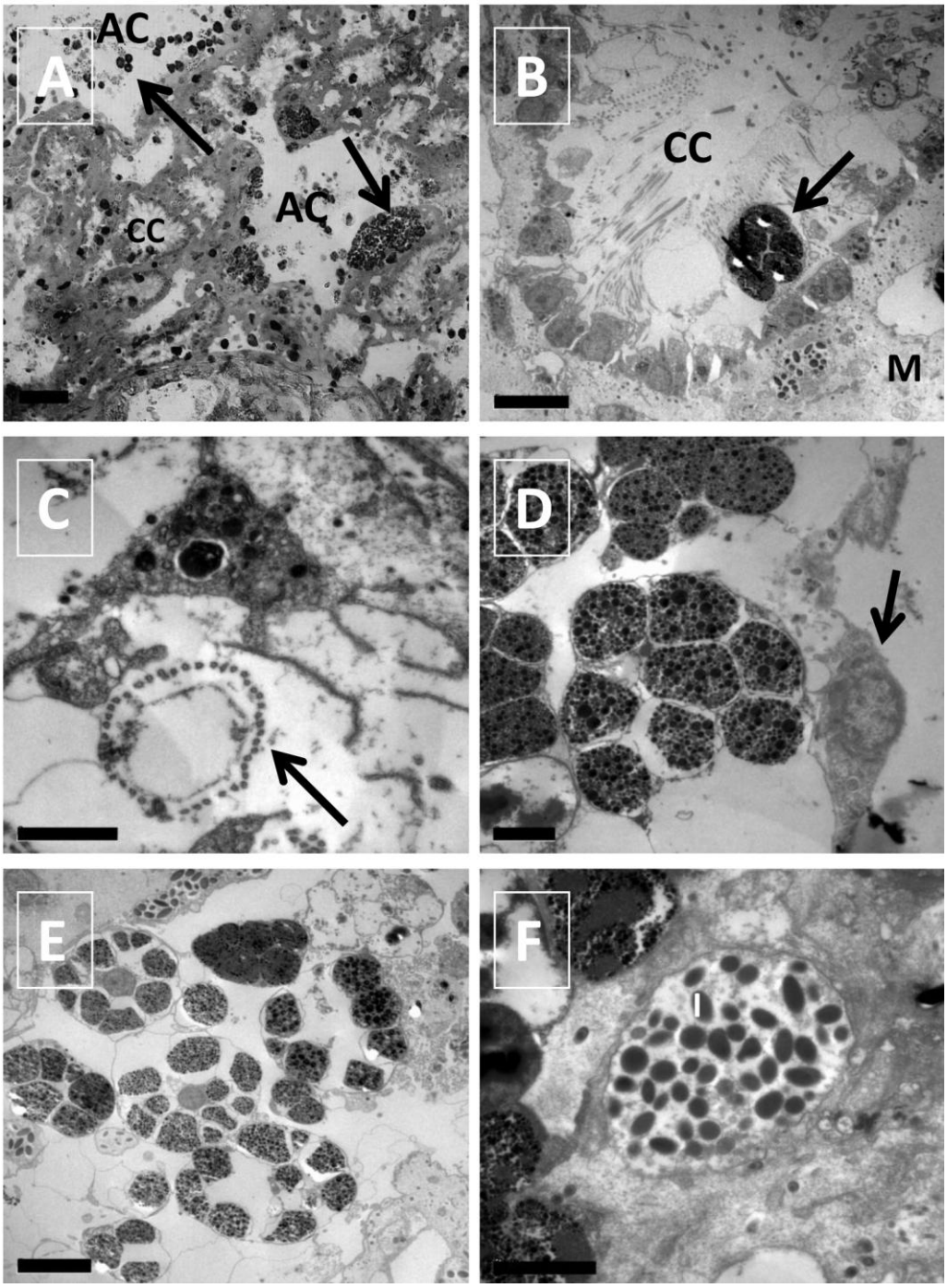

Figure 6: A- Semi-thin section through *H. crypta*. B-F: TEM views of *H. crypta*. A-The choanosome with abundant individual and aggregated cells with inclusions (arrows). Aquiferous canals (*AC*) are of variable size surrounded by flagellated choanocyte chambers (*CC*) B- View of a large, eurypylous choanocyte chamber (*CC*) containing a spherulous cell with large inclusions containing microgranules (type 2, arrow). Notice the microgranular cell lying in the mesohyl (*M*). C- Choanocyte, cross section trough the microvilli (arrow) composing the choanocyte collar. D- Some spherulous cells type 2, lying close to a pinacocyte (arrow). E- Evolutionary stages of spherulous cells type 2, mainly some degrading forms, with gradual increase of cytoplasm volume. F-Detail of a microgranular cell (type 3) containing osmiophile inclusions (*I*), the section does not include the nucleus. Scale bars: A= 25µm B=5µm, C= 2µm, D= 2µm, E=5µm, F=2µm.

Hexadella racovitzai (Topsent 1896)

(Figure 7)

Holotype : MNHN xx (only slide preparation of preserved material, courtesy of N. Boury-Esnault). Shallow water, Melobésiées du Cap Abeille, Banyuls-sur-Mer, France, Western Mediterranean France (Bergquist and Cook, 2002).

Material examined

Paratype: MNHN xx: Specimen 8 Tombant du Loews, Monaco, France (35 m depth)

Other material examined

Marseille : two specimens (Specimen 4 and 5) from Impérial de Terre (depth 25m) and two specimens (Specimen 26 and 27) from Tiboulen du Frioul (depth 38 m). **Monaco:** three specimens (Specimen 7, 9, 10) from Tombant du Loews (depth 35 m)

GenBank Acc.number:FN667718 (corresponding to haplotype R15mam, see Reveillaud et al., 2010).

Diagnosis

Faded to pale pink *Hexadella* in vivo, changing to brownish in ethanol. Surface covered with a prominent star-shaped network of canals converging towards the wide oscula. Contains two types of inclusion cells: spherulous cells with large inclusions containing microgranules (type 2) and microgranular cells (type 3). Choanocyte chambers (± 30 x 19,1) µm. Exhibits a rather high natural toxicity.

Description of MNHN xx

The sponge consists of a thin crust with a lobate shape. The surface is highly wrinkled with small parallel or reticulate stripes, while pores are microscopic. The surface is almost entirely covered with tube shaped membranes (i.e., canals) forming star-shaped networks leading to large openings on top, the oscula (Fig. 3C). Color of living specimens is faded to pale pink. Its color becomes brownish in ethanol, and the fixative gets a yellow tint. We note that the membrane-like exhalant canals are more developed than in *H. pruvoti* and *H. crypta.*

General organisation

The ectosome forms a film of noticeable thickness. In contrast, the choanosome is soft, fleshy and fragile, difficult to cut. The choanosome contains densely packed eurypylous choanocyte chambers of (30 ± 6.3) x (19 ± 2) µm on average (Fig. 7A-B). Large aggregates of cells with inclusions (Fig. 7A) are present in the mesohyl.

Cytology

Choanocytes are spherical to ovoid, (3.6 ± 0.6) x (2.7 ± 0.6) µm on average and laterally bounded to one another (Fig. 7C). They are anchored in the mesohyl through their short basal pseudopodia. The nucleus, (2.0 ± 0.3) x (1.7 ± 0.3) µm in size is located centrally and is nucleolated (size of (0.6 ± 0.2) x (0.5 ± 0.3) µm). A wide cytoplasmic collar surrounds the choanocyte. Under the choanocyte line, the mesohyl contains small rod-like bacteria ± 0.5 µm in length (Fig. 7C, arrow). Two different types of cells with inclusions are distinguishable within the mesohyl. The dominant cells with inclusions are of type 2, spherulous, approximately (8.1 ± 1.8) x (5.8 ± 4.0) with large inclusions containing microgranules and an anucleolated nucleus (Fig. 7D-E). They can form packages or occur individually and are mainly present in the mesohyl, although they were also observed within the choanocyte chambers (Fig. 7E). The microgranules within the inclusions show variable density between cells, probably reflecting different stages of cell development. Occasionally some density variations are also observed within the cells (Fig. 7D). Some lateral expansions of the cytoplasm may be present. These cells are generally bordered by pinacocytes (Fig. 7D). Microgranular cells (type 3; Fig. 7F) are rare but observed within the mesohyl. They are irregular in shape, (5.6 ± 0.9) x (4.5 ± 0.6) µm in size and contain a small nucleus. Oval to rod-like dense osmiophilic granules (approximately 10-30 per cell) are randomly distributed within the cell.

Chemistry

As a first result, we obtained HPLC traces with well observable (0.01 – 1.5 AU) and resolved peaks corresponding to the chemical fingerprints of each crude extracts of *H. racovitzai* individuals (Specimen 4, 5, 7, 8, 9 and 10). The six chemical fingerprints formed a homogeneous group in which all specimens showed one peak in the polar compounds' zone (3-5 min) at 4.0 min and two intense peaks in the medium polar compounds' zone (15-22 min) at 16.5 and 18.6 min, together with some minor peaks (5.1, 17.7, and 20.2 min). Among

the homogenous group of chemical fingerprint for the species, the chromatogram of the Specimen 10 extract was chosen as representative and shown in Fig.5 (A). Comparison of the UV spectra of the three major compounds from all the six individuals clearly confirmed the homogeneity of this group. The specimens differed only by the relative intensity of major and minor peaks. Specimen 8 was distinguishable from others by an under-expression of peak at 16.5 min. The same observations could be made by comparison of Max Plot chromatograms (data not shown).

Natural toxicity quantification

Crude extracts of *H. racovitzai* (Specimen 4, 5, 7, 8 and 10) formed a homogenous group showing very low EC_{50} values (from 1 to 5 µg/mL), which means a very high natural toxicity.

Locality and habitat

All the examined material was collected in habitats shaded from light, a rather deep coralligenous overhanging cliff and semi-dark submarine caves, from 25 to 38 m. However this sponge has already been observed deeper than 100 m (Port-Cros, 180 m, Reveillaud et al., 2010 and unpublished observations). For now we can only confirm its distribution in the North Western Mediterranean Sea, but studies that were performed before the analysis of the intra-interspecific molecular variation for the taxa, reported also *H. racovitzai* from the Canary Islands (Cruz, 1985), the Azores (Boury-Esnault and Lopez, 1985), Roscoff (Borojevic et al., 1968), Madagascar (Vacelet et al., 1976), South of Portugal (Xavier and van Soest, 2007) and the British Isles (Morrow and Picton, 1996; Picton and Costello, 1998; Picton and Goodwin, 2007).

Taxonomic remarks

Because the original description of Topsent (1896) reports a faded to pale pink color, the specimens which in this study exhibited the same shape were all considered as *H. racovitzai* sensu stricto.

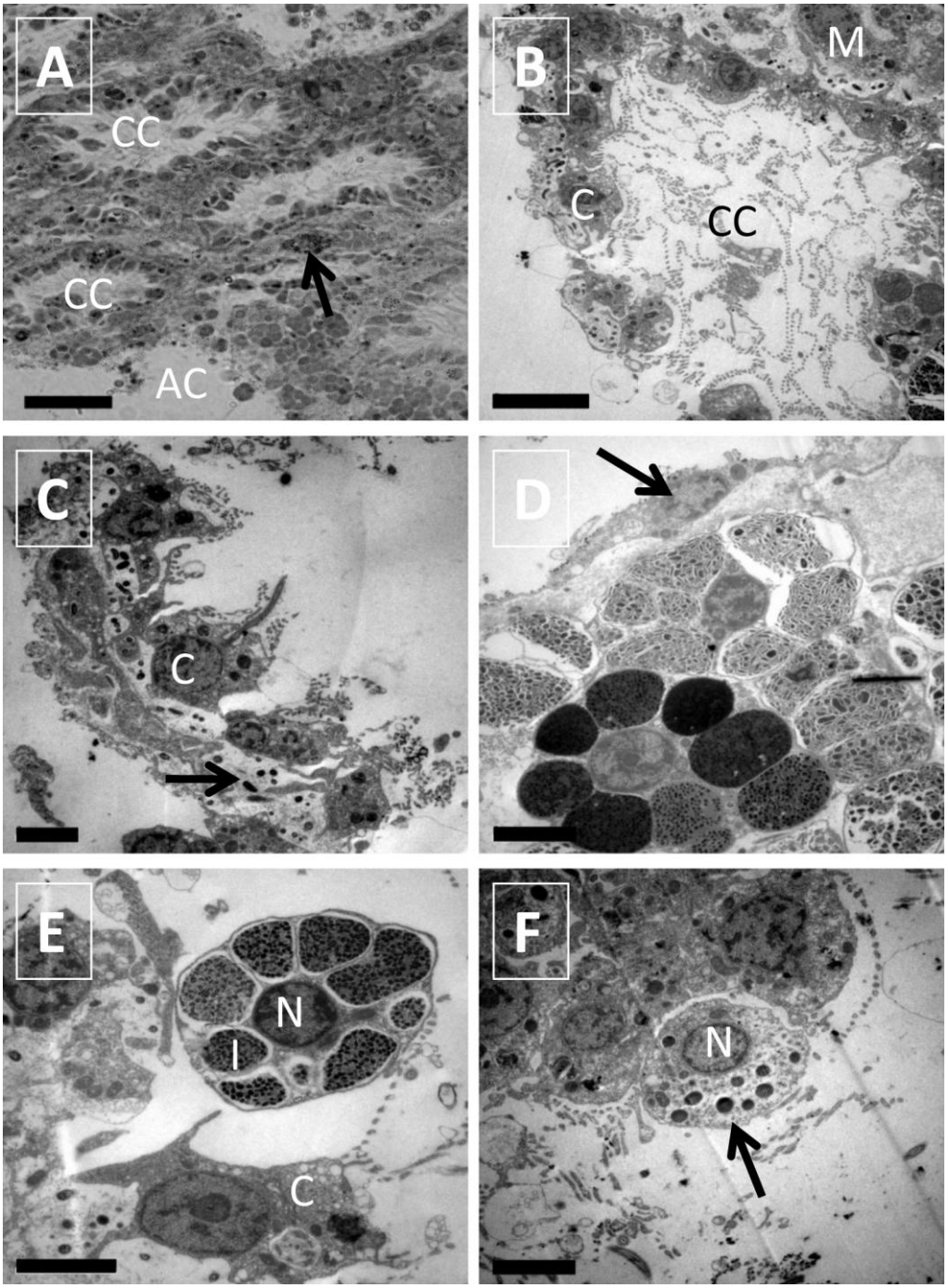

Figure 7: A: Semi-thin section through *H. racovitzai*. B-F TEM views of *H. racovitzai*. A- The choanosome, with large, sac shaped, eurypylous, choanocyte chambers (*CC*), arranged around the aquiferous canals (*AC*). Cells with inclusions (arrows) are largely abundant, around the aquiferous canals, within and between the choanocyte chambers. B- Choanocyte chamber (*CC*), with its choanocytes (*C*). The chamber is surrounded by mesohyl (*M*). C- View of some choanocytes (*C*), surrounded by mesohyl containing bacteria (arrow). D- Spherulous cells type 2, in different forms of maturation, containing large inclusions with granular content. Notice the pinacocytes (arrow) bordering these cells. E- A spherulous cell, its nucleus (*N*) and granular inclusions (*I*) within a choanocyte chamber, with a choanocyte (*C*). F- Microgranular cell (type 3) with its nucleus *(N)* and small spherulous to rod-shaped inclusions (arrow). Scale bars: A=12,5µm, B= 5µm, C=2µm, D=2µm, E=2µm, F=2µm

Hexadella topsenti sp. nov.

(Figure 8)

Restricted synonymy. *Hexadella racovitzai*

Material examined

Holotype. MNHN xx. Specimen 1, Impérial de Terre, Marseille, France (depth 25m).

Paratypes. **Marseille:** one specimen (Spe 2) from Impérial de Terrre (43°10.37'N, 05°23.58'E, 25m depth) and three specimens (Spe 14, 15, 16) from Grotte à Corail (43°12.36'N, 05°19.57'E, 10m depth).

GenBank Acc.number: FN667719 (corresponding to haplotype R16mam, see Reveillaud et al., 2010).

Diagnosis

Bright to dark pink *Hexadella* when alive, changing to brownish in ethanol. Surface with some small, chimney-like protrusions, converging towards the oscula. Contains two types of inclusion cells: spherulous cells with large inclusions containing microgranules (type 2) and microgranular cells (type 3). Choanocyte chambers (35 X 20) µm. Low to moderate natural toxicity

Description of MNHN xx

The sponge is thinly encrusting with lobate body. Living specimens are bright to dark pink and even purple. In ethanol, it turns brownish, while the fixative takes a yellow tint. The surface is smooth but striated, with tiny conules connected by a reticulation of thin ridges. Foreign bodies are present. Pores are microscopic and oscules are abundant, scattered, resembling small chimneys, as they are the prolongation of membrane-like surface canals (Fig. 3D).

General organisation

The ectosome contains some bundles of collagen fibrils. The body is unsupported and lacunose. The choanosome displays large choanocyte chambers of (35 ± 7) x (20 ± 0) µm

diameter, a reduced mesohyl and a developed lacunar system (Fig. 8A). Large aggregates of cells with inclusions (Fig. 8A) and rod-shaped bacteria (Fig. 8D) are present in the mesohyl.

Cytology

Choanocytes (Fig. 8B) are spherical to subspherical, (4.6 ± 0.9) x (3.2 ± 0. 7) µm, laterally bounded to one another and anchored in the mesohyl through their short basal pseudopodia. Their nucleus, (2.3 ± 0.4) x (2.0 ± 0.3) µm on average, is centrally located and nucleolated (0.7 ± 0) x (0.6 ± 0.1) µm. A wide cytoplasmic collar surrounds the choanocyte. Pinacocytes are flattened, of about (4.3 ± 3.2) x (1.6 ± 1.4) µm, with long pseudopodia at both ends and a nucleolate (0.7 ± 0.3) x (0.6 ± 0.1) µm nucleus of [(2.3 ± 1.3) x (1.8 ± 0.9)] µm occupying almost the entire cell. Archaeocytes (Fig. 8C) measure [(5.3 ± 2) x (3.6 ± 1.4)] µm, and contain a large nucleole of about (0.8 ± 0.2) x (0.7 ± 0.3) µm with the nucleus of (2.6 ± 0.6) x (2.3 ± 0.7) µm on average. They can be distinguished from other cell types by their large nucleolate nucleus and by the presence of some phagosomes. Two different types of cells with inclusions are distinguished. Spherulous cells with large inclusions containing microgranules (type 2; Fig. 8B, 8E), are highly abundant, and tend to form packages but also occur individually. They are present in the mesohyl, sometimes in the choanocyte chambers and can be found concentrated around canals (Fig. 8A). They are round to ovoid, (7.2 ± 2.3) x (5.5 ± 1.7) µm, and they contain an anucleolate nucleus. The cytoplasm may show some expansions (see the arrow on Fig. 8E). These cells seem to originate from totipotent archaeocytes, and different stages of evolution can be observed from early formation to degenerescence. Microgranular cells (type 3, Fig. 8F) are rare and randomly distributed throughout the mesohyl. They are irregular in shape, (5.8 ± 1.4) x (3.8 ± 1.7) µm on average. They have a small nucleus and contain oval to rod-like osmiophilic dense granules (about 10-30 per cell).

Chemistry

We obtained HPLC traces with well observable (0.01 – 0.7 AU) peaks corresponding to the chemical fingerprints of each crude extract of *Hexadella topsenti* individuals (Specimen 1, 2, 14, 15). The four chemical fingerprints showed chromatograms with one peak in the polar compounds' zone (4,0 min) and peaks in the medium polar compounds' zone (17-22 min). The chromatogram of the Spe 2 extract (B, Fig. 5) was chosen as the representative chemical fingerprint for the species as it showed the sharper and more intense peaks. A broad and asymmetrical peak, common for all *H. topsenti* specimens (4/4) was present in the zone

between 18 and 19.5 min. Two intense peaks at 19.8 and 20.5 min were observable in chromatograms of specimens 1 and 2. These two peaks were replaced in specimens 14 and 15 by an unresolved peak (19,1-20,6 min) with inside at least four compounds. All the intense peaks (specimens 1, 2, 14, 15) in this zone (19 to 20,5 min) possessed the same UV spectrum indicating that they are structurally related. Comparison of Max Plot chromatograms (data not shown) also led to the conclusions that the four chemical fingerprints formed a uniform group of specimens.

Natural toxicity quantification

Crude extracts of *H. topsenti* (Specimen 1, 2, 14, 15) exhibited EC_{50} values ranging between 139 and 287µg/mL which correspond to a low to moderate natural toxicity.

Locality and habitat

The type material was collected from a coralligenous cliff in Marseille (France, NW Mediterranean). This species is usually more exposed to light than *H. racovitzai* s.s. Other representatives from rocky substrates of the NW Mediterranean were studied (Banyuls, 35 m; Grotte à Corail Marseille, 10m, Impérial de Terre, 25m, this study and Reveillaud et al., 2010, Corsica 15-40 m). The sponge has also confirmed records in the NE Atlantic (Gorringe bank, South Portugal, 32m; Plymouth, England, 30m and Rathlin Island, Northern Ireland, 30m; Reveillaud et al., 2010) and we may hypothesize that this species is widely distributed throughout the Mediterranean.

Etymology

The species is dedicated to Émile Topsent who first described the genus *Hexadella*.

Taxonomic remark

This species shows a clearly brighter and deeper pink color than *H. racovitzai*. It also can be recognized through its small chimney-like protrusions, converging towards the oscula. At the cytological level, choanocytes are significantly larger in *H. topsenti* than in *H. racovitzai* ($p<0.001$, $H=17.04$, $N=66$). Already in 1996, Morrow and Picton observed the existence of deep-salmon pink specimens (possibly *H. topsenti*) to be possibly distinct from pale-yellow-cream *H. racovitzai*-like specimens.

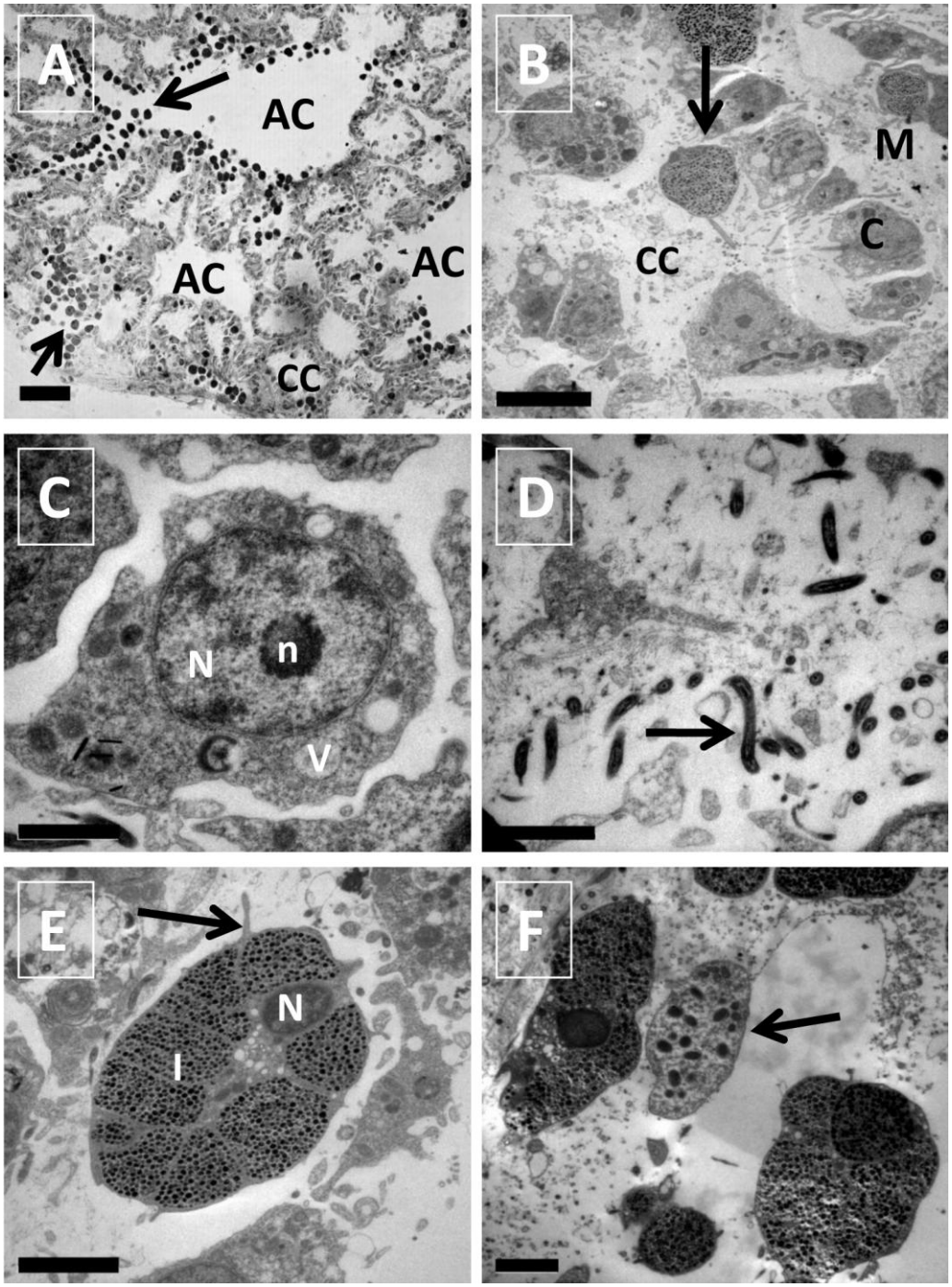

Figure 8: A, Semi-thin section trough *H. topsenti*. B-F TEM views of *H. topsenti*. A- Choanosome with its aquiferous canals (*AC*) surrounded by large eurypylous choanocyte chambers (CC). The choanosome contains a large amount of cells with inclusions (arrows), distributed throughout the mesohyl, within choanocyte chambers but mainly concentrated around the aquiferous canals. B- Choanocyte chamber (*CC*), with its choanocytes (*C)*. The chamber is surrounded by mesohyl (*M*) and contains a spherulous cell type 2 (arrow). C- View of an archaeocyte, with large nucleolated (*n*) nucleus (*N*) and vacuoles (V) within the cytoplasm D- View of bacteria in the mesohyl (arrow). E- Detail of a spherulous cell type 2, containing large inclusions (*I*) with granular content. Notice the lateral extensions of the cytoplasm (arrow). F- Microgranular cell (type 3, arrow), the section does not show its nucleus. Scale bar: A=25µm B= 5µm, C=1µm, D=1µm, E=2µm, F=2µm.

Discussion

In the present study, phylogenetic reconstructions based on the COI mitochondrial marker confirmed the presence of two divergent lineages within both *H. pruvoti* and *H. racovitzai*. Within each taxon, the two genetically separated group of specimens share a large number of phenotypic and habitat features, so that it is difficult to distinguish them. All species are thin encrustations, inhabiting shallow hard substrate communities, which can be observed in sympatry in the North Western Mediterranean Sea (Marseille area), thus increasing the difficulty to discriminate the taxa. Although *H. pruvoti* and *H. racovitzai* were already recorded from the deep Mediterranean Sea, only the report of *H. racovitzai* at 180 m in Port-Cros has been confirmed by a molecular study (Reveillaud et al., 2010). Topsent's ancient description of both *H. racovitzai* and *H. pruvoti* was brief. Therefore it was difficult to correctly link the recorded haplotypes with the original species described by Topsent. Nevertheless, a close examination of the four species together allowed recovering some consistent diagnostic differences (cf Table 2). Steady in vivo-color differences are found between *H. racovitzai* and *H. topsenti* individuals. As originally observed by Topsent, *H. racovitzai* is consistently faded, pale pink, while *H. topsenti* sp. nov. is always bright pink to purple. In addition, both species can be distinguished based on the appearance of their surface aquiferous canal system. All *H. racovitzai* individuals constantly show a prominent star shaped network of canals covering the entire sponge surface. In contrast, *H. topsenti* has a poorly defined network, but some membrane-like "chimneys" leading to the oscula are observed on their body surface. It is noteworthy that both species do not turn purple in ethanol but rather take a brownish color, while they give a faded and yellow tint to the fluid after several hours (De Laubenfels, 1950; this study). At the cytological level, *H. racovitzai* and *H. topsenti* have similar cell types with overlapping cell size ranges ($p>0.05$). Only choanocytes are significantly larger in *H. topsenti* than in *H. racovitzai* ($p<0.001$). To survive on crowded and complex ecosystems, each sponge species produces a different set of secondary metabolites that help them to occupy their particular niche. These compounds can display specificity on different taxonomic levels (i.e., from species to phylum) and be used as taxonomic characters. *H. racovitzai* and *H. topsenti* can be separated based on distinct metabolic fingerprints. *H. racovitzai* individuals showed a chromatogram characterized by the presence of two intense peaks at 16.5 and 18.6 min (Figure 5A). *H. topsenti* specimens shared a common compound with *H. racovitzai* (same retention times and UV spectra) at 16.5 min but differ by the presence of intense peaks at 19.8 and 20.5 min (Figure 5B) instead of the one

at 18.6 min. A putative consequence of the distinct metabolic fingerprint is the significant difference between natural toxicity of crude extracts of both species, *H. racovitzai* being 50 to 100 times more toxic than *H. topsenti*.

Table 2: Main ecological, morphological, cytological and chemical differences between the four valid *Hexadella* species (numbers of specimens studied indicated in parenthesis).

	H. racovitzai (8)	***H. topsenti (5)***	***H. pruvoti (4)***	***H. crypta (10)***
Depth (m)	25-180	10-35	10-35	10-42
Color *in vivo*	Faded, pale pink	Bright to dark pink	Bright yellow	Bright yellow
Color in contact with ethanol	Brownish	Brownish	Purple	Purple
Color of ethanol after sample fixation	Yellow	Yellow	Yellow	Dark purple
Surface	Wrinkled with prominent membrane-like, star shaped canals leading to oscula	Wrinkled with prominent oscula	Wrinkled with some rare oscula	Wrinkled with some rare oscula
Shape	Encrusting, thin, lobate	Encrusting, thin, lobate	Encrusting, thin, lobate	Cushion like encrustation, moderate thickness, non lobate
Average size (µm) choanocyte chamber	[(30 ± 6,3) x (19,1 ± 2)]	[(35 ± 7)x (20 ± 0)]	(40x20)	(30x20)
Average size (µm) choanocyte	[(3,6 ± 0,6) x (2,7 ± 0,6)]	[(4,6 ± 0,9) x (3,2 ± 0,7)]	[(4,3 ± 0,6) x (3,5 ± 0,7)]	[(3,9 ± 0,7) x (3,1 ± 0,6)]
Average size (µm) archaeocyte	Not observed	[(5,3 ± 2) x (3,6 ± 1,4)]	Not observed	Not observed
Average size (µm) pinacocyte	Not observed	[(4,3 ± 3,2) x (1,6 ± 1,4)]	(5,5 x 2,8)	(8,5x3,2)
Average size (µm) spherulous cell with large granular inclusions (type 1)	Absent	Absent	[(11,2 ± 4,7) x (7,7 ± 3,1)]	Absent
Average size (µm) spherulous cell with large inclusions containing microgranules (type 2)	[(8,1 ± 1,9) x (5,8 ± 4)]	[(7,2 ± 2,3) x (5,5 ± 1,7)]	[(8,9 ± 1,9) x (5,4 ± 1,4)]	[(8,9 ± 2,3) x (6,7 ± 1,7)]
Average size (µm)microgranular cell (type 3)	[(5,6 ± 0,9) x (4,5 ± 0,6)]	[(5,8 ± 1,4) x (3,8 ± 1,7)]	[(7,3 ± 0,9) x (4,6 ± 1)]	[(6,2 ± 2,1) x (3,8 ± 1)]
Present secondary metabolites	Group A	Group B	Group C	Group D
Toxicity	Very high	Low	Medium	Medium

Morphological discrimination between *H. pruvoti* and *H. crypta* is highly challenging as no inter-species variation based on color *in vivo* or on the surface aquiferous system could be observed. However, *H. crypta* recurrently shows a less diffuse, less encrusting and slightly thicker growth habitus compared to *H. pruvoti*. In addition, although both species turn dark purple in ethanol, *H. crypta* individuals tend to give to the fixative the same purple color, while *H. pruvoti* individuals give a yellow tint to the liquid. Interestingly, the morphometric study of *Hexadella* cell types showed that *H. pruvoti* and *H. crypta* differ in cellular content. Both species shared spherulous cells with large inclusions containing microgranules (type 2) and microgranular cells (type 3). However, the spherulous cells with large granular inclusions (type 1) are exclusively present in *H. pruvoti* and never found in *H. crypta* specimens. Nicole Boury-Esnault (personal communication) also observed these spherulous cells type 1 in some unpublished photographs of the former *H. pruvoti* specimens.

The comparative analysis of HPLC chromatograms of *H. pruvoti*-like specimens showed different chemical fingerprints from that of *H. racovitzai*-like specimens with a higher molecular diversity for the *H. pruvoti*-like specimens essentially in the polar compounds' zone (3-10 min). Furthermore, *Hexadella pruvoti* (Figure 5C) and *H. crypta* (Figure 5D) showed two different chemical profiles. The two species share common "polar" compounds (4.1, 6.0 and 7.4 min) but show clearly specific signatures in the medium polar compounds' zone. The UV spectra of the "polar" compounds are closely related to those of aerophobins 1 and 2, two bromotyrosine alkaloids isolated in *Aplysina* species, whereas the UV spectra of the series of peaks, specific to *H. crypta* in the medium polar zone (12-22 min), indicate that these compounds belong to the fistularin family, a family of bromotyrosine metabolites characteristic of *Aplysina aerophoba*. Therefore, *H. pruvoti* and *H. crypta* may to some extent be closer to *Aplysina* species than to *H. racovitzai*-like specimens in terms of secondary metabolites.

In sponges, all characters, from color to spicule size may show a certain plasticity and therefore may present high levels of intra-specific variability (Klautau et al., 1999). Color is often considered as an ambiguous character in sponge taxonomy as it can be prone to environmental changes (light for instance). Therefore, depending on the author' s view, *H. racovitzai* and *H. pruvoti* have been seen as two distinct species showing significant color differences (Topsent 1896, Vacelet 1969, Morrow and Picton 1996), or alternatively as

conspecific species with a high degree of intra-specific color variation (De Laubenfels, 1950). Nevertheless, some authors showed that color variation is congruent with genetic variation in some sponge species (Blanquer and Uriz, 2008) as well as in other Phyla (e.g Echinodermata; Williams, 2000). This study further illustrates that when the intra- and interspecific character variation is studied and integrated with other diagnostic characters (e.g. chemical and molecular data), the discrimination of species gains reliability. Here, outer **morphology** and color proved to be operative in discriminating the four species; *in vivo* color and aspects of surface structures allowed separating *H. racovitzai* from *H. topsenti*. Similarly, sponge growth habitus and different tints of the ethanol after storing the material showed to be highly variable between the species *H. pruvoti* and *H.crypta*. These easily accessible diagnostic features should allow a quick identification of the four species. In addition, this work adds to the growing evidence that pigment can help in the species identification of keratose species (Bergquist 1980). The characteristic oxidation reaction upon damage or death in Verongids, turning the yellow species to a final dark purple coloration has been recurrently described for *Hexadella* species (Topsent, 1913; De Laubenfeld, 1950; Vacelet, 1969, Xavier and Van Soest 2007; Reveillaud et al., 2010). This study suggests that the ethanol reaction itself can be useful for sponge systematics.

Cytology, particularly sponge cell categories, showed to be effective in the separation of *H. pruvoti* and *H. crypta*, the former holding uniquely three types of cells with inclusions. On the other hand, *H. racovitzai* and *H. topsenti* were not discernable based on cytological information. The function(s) of cells with inclusions is (are) not yet defined (Vacelet, 1967; Ereskovsky et al., 2009). However, TEM observations in this study showed multiple developing and degenerating stages of cells with inclusions. A regularly-based sampling could provide new insights into the life cycle of these cells and help resolving the question of their diversity. Nevertheless, this study confirmed that cell ultrastructure descriptions and the comparison of cell types can successfully resolve a few taxa, as pointed out previously (Boury-Esnault et al., 1995; Muricy et al., 1996). In these former studies and confirmed for *H. pruvoti*-like specimens, mesohylar cells with inclusions provided good diagnostic features at the species level.

The structural complexity of the **secondary metabolites** has suggested this large source of characters as an alternative to morphological characters (see for instance Van Soest

and Braekman, 1999; Ivanisevic et al., 2011). Here, sponge chemotaxonomy was used with promising results for the four *Hexadella* species and showed coherence with DNA analysis. Although these results have no known ecological meaning in themselves, the clear differentiation between 1- *H. racovitzai*-like and *H. pruvoti*-like specimens and, 2) *H. racovitzai* and *H. topsenti* was supported by measurements of natural **toxicity** and the synergy of both makes the total more robust. The similar natural toxicity values between *H. pruvoti* and *H. crypta* further emphasizes the need to study independent diagnostic characters in an integrative way for the delineation of species.

This study indeed highlights the great potential of an integrative systematic approach in resolving systematics of small and encrusting sponge species which lack skeleton. In 1859, Darwin emphasized that *"…a classification founded on any single character, however important that may be, has always failed"*. As a matter of fact, any kind of newly discovered characteristic is equally good, as long as it shows stable differences whatever the number of replicates is. However, as shown in this study, each taxonomic character taken apart can not reveal significant differences for all *Hexadella* species. Although time consuming, the concert of methods applied in our study allowed to point out differential features between cryptic species. Thus, it demonstrates that the analysis of a great number of specimens together in a comparative way enhances taxonomic diagnoses, and therefore can provide robust and rigorous classifications.

Phylogenetic reconstructions based on the congruence between independent nuclear and mitochondrial markers (Reveillaud et al., 2010) suggested the occurrence of an *H. dedritifera* species complex, with *H. dedritifera* sensu stricto in the Northern deep-sea latitudes, *H. cf. dedritifera* in the deep-sea Mediterranean Sea, and a putative new *Hexadella* species associated to deep-water corals in the Ionian Sea. These DNA analyses also showed representatives of *H. pruvoti* to be possibly more closely related to the deep-sea *H. cf. dedritifera* than to *H. crypta*. The formal description of these genetically separated species would require new deep-sea sampling campaigns, and the comparison of these deep-sea cryptic species with the four species presently analyzed. Nevertheless, noteworthy is the fact that Topsent measured much larger choanocyte chambers (ranging from 107 x 60 to 165 X 73 µm) in the deep-sea *H. dedritifera* than in both shallow *H. racovitzai* and *H. pruvoti* (75 µm at max for the largest axis, Topsent, 1913). The choanocyte chamber measurements of the four

species performed in this study did not exceed 40 X 20 µm diameter (cf Table2), and are hence in agreement with the description of Topsent.

To conclude, this study emphasizes the usefulness of complementary methods to be plotted against a robust and comprehensive phylogenetic (DNA) analysis in skeleton-free sponge diversity studies, as it allowed to detect four *Hexadella* species, which were originally considered as two species, and to accompany these new species by a sound taxonomical description. It is obvious that genetic information should be an active compound of today's systematics, allowing to detect cryptic species, to shed light on true levels of diversity in the marine environment, and to reconstruct sponges phylogeny. However, only a formal description will ensure access to the species, and therefore make the best use of molecular discoveries. This work promotes the use of all available scientific resources to give meaningful species description and delimitation between taxa, and even to highlight some hidden biodiversity.

Acknowledgment

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CHAPTER V

Phylogenetic Relationships among NE Atlantic Plocamionida Topsent (1927) (Porifera, Poecilosclerida): Under-Estimated Diversity in Reef Ecosystems

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CHAPTER V

Abstract

Background: Small and cryptic sponges associated with cold-water coral reefs are particularly numerous and challenging to identify, but their ecological and biochemical importance is likely to compete with megabenthic specimens.

Methodology/Principal Findings: Here we use a combination of the standard M1M6 and I3M11 partitions of the COI fragment, partial 28S rDNA sequences and morphology to delineate small encrusting *Plocamionida* species. In total, 46 specimens were retrieved from seven shallow to deep-water coral locations, crossing 3,000 km along the European margins. Our work provides evidence that the *Plocamionida ambigua f. tylotata* and *f. grandichelata* can be considered valid species, whereas *Plocamionida ambigua f. tornata* corresponds to the species *P. ambigua*. Within the monophyletic group of *Plocamionida*, *P. microcionides* is shown as really divergent from the other taxa, and four putative new *Plocamionida* species are suggested.

Conclusions/Significance: This study shows that the use of molecular and morphological information in an integrative approach is a powerful tool for the identification of sponge species, and suggests that an under-estimated biodiversity of sponges occurs in cold-water coral reefs.

Keywords: Phylogeny; Porifera; Cold-water coral; COI; Mitochondrial DNA; Partial 28S rDNA

Introduction

Sponges represent one of the most remarkable[16] groups in deep-water coral ecosystems (Jensen and Frederiksen, 1992; Rogers, 1999). The high biodiversity and abundance of these filter-feeders (with a total of 191 species recorded in Irish bathyal coral reefs, Van Soest et al., 2007a) contrasts with the paucity of the coral reef building species, predominantly *Lophelia pertusa* (Linnaeus 1758) and *Madrepora oculata* (Linnaeus 1758). Ecologists initially focused on large-sized, bright-coloured or conspicuous Porifera species (e.g., Rice et al., 1990; Barthel, 1991; Klitgaard and Tendal, 2004)), but the extensive presence of small sized and morphologically cryptic sponges in cold-water coral reefs (CWRs) has widely been noted (Vacelet, 1969; Longo et al., 2005; Van Soest and Lavaleye, 2005; Van Soest et al., 2007a; Reveillaud et al., 2010). Single dead coral branches from cold waters can contain up to 15 sponge species (Van Soest and Lavaleye, 2005). The distribution of CWRs along the European margins has now been well documented (Roberts et al., 2006), and several coral hotspots are found along the continental margin off Ireland, off Norway, and in the Mediterranean basin. Nevertheless, only few biodiversity studies have addressed the substantial diversity of deep-sea sponges associated with CWRs (Longo et al., 2005; Van Soest and Lavaleye, 2005; Van Soest et al., 2007a; Reveillaud et al., 2010). Such lack of knowledge forms a substantial impediment for establishing baselines of biodiversity and for the efficient management of this group (Van Soest and Lavaleye, 2005), which is of particular interest for the pharmaceutical industry (Faulkner, 2000). Moreover, the significance of these deep and nutrient rich hotspot ecosystems for potential centers of endemism has direct implications for both regional diversity and local endemicity.

The sponge genus *Plocamionida* Topsent (1927) (Class Demospongiae, Order Poecilosclerida, Family Hymedesmiidae) is widely distributed along the continental margins of Europe, and occurs from the Mediterranean Sea and the Azores to ‘‘high latitudes’’ in the NE Atlantic (Stephens, 1921; Topsent, 1928; Boury-Esnault et al., 1994; Van Soest and Lavaleye, 2005). Within CWR environments, *Plocamionida* species encrust rocks or hard corals in thin (<5mm) sheets of brown coloration and can be locally abundant. Occasionally, shallow-water occurrence is reported, but their main occurrence is at depths of 50 m and

[16] Species-rich

deeper. Although the genus has excellent morphological markers, the taxonomic distinctness of its European species remains highly contentious. Two species names, *P. ambigua* (Bowerbank, 1866) and *P. microcionides* (Carter, 1876) have been considered as valid separate species, or as synonyms of a single variable species (e.g., Stephens, 1921; Topsent, 1928). Furthermore, a number of 'formas' have been proposed by various authors for specimens with deviating spicule characteristics, *Plocamionida microcionides f. achelata* Topsent, 1928, *Plocamionida ambigua f. grandichelata* Brøndsted, 1932, *Plocamionida ambigua f. tornotata* Brøndsted, 1932 and *Plocamionida ambigua f. tylotata* Brøndsted, 1932. The latter form has been given species status by Alander (1942) and Picton and Goodwin (2007). *Plocamionida* remains a group of sponges that are notoriously difficult to identify because the intra- and interspecific character variation is not well understood, and has given rise to disagreements between taxonomic experts.

The first aim of this study is to investigate the phylogenetic relationships of *Plocamionida* species and formas from the Gulf of Cadiz to Norway occurring in CWRs. Specimens from one shallow water population were also included. We used phylogenetic congruence criteria between the cytochrome oxidase c subunit I (COI) and the independent nuclear region D3-D5 of the rDNA 28S gene to delineate evolutionary significant units, and to reveal the presence of cryptic species within the studied material. The standard (COI) barcoding fragment, amplified with the universal primers of Folmer et al. (1994; hereafter called the M1M6 partition) is generally too conserved in diploblast phyla (Shearer et al., 2002) and has led to some difficulties in resolving taxonomic and phylogeographic relationships in sponges (Duran et al., 2004; Wörheide, 2006). On the other hand, genetic studies above and below the species level have been performed using this COI partition (Erpenbeck et al., 2002; Duran and Rutzler, 2006; Wulff, 2006; Blanquer and Uriz, 2007; Reveillaud et al., 2010). In addition, the COI downstream I3M11 partition showed more resolution than the standard M1M6 partition (Erpenbeck et al., 2006b). It proved useful at interspecific level (Xavier et al., 2010a) and to determine the genetic population structure of Caribbean and European sponge species (Lopez-Legentil and Pawlik, 2009; Xavier et al., 2010b). Herein, we seize the opportunity to compare the I3M11 partition to the M1M6 partition in terms of amplification success and substitution pattern, as well as to assess the potential of their combination for species level delineation. Second, the phylogenetic units

were morphologically analyzed to investigate whether concordant molecular lineages are also morphologically distinct and to resolve the current taxonomic difficulties in *Plocamionida*.

Material and methods

Sampling

A total of 46 specimens of *Plocamionida* were collected from seven locations along the Atlantic continental margin spanning about 3,000 km (Fig. 1). Normal storm waves disturb the seafloor significantly down to 50m (Lindner et al., 2008) and this depth was used to separate shallow from deep-water environments. Deep-water specimens (>50m) were collected with boxcores or with a Remote Operated Vehicle (ROV) during five cruises and one specimen was dredged up from the coast of Norway in Bergen (Table S1). Shallow-water specimens were collected by scuba diving at The Maidens, Northern Ireland. Specimens were detected by searching dead coral branches and stones using a low power microscope. All samples were preserved in absolute ethanol and stored at room temperature until further processing. *Plocamionida*-like individuals were identified by looking at their skeletal structure using thick sections air-dried on microscopical slides and mounted in Canada balsam. Voucher specimens are deposited in the Porifera collection of the Zoological Museum of Amsterdam (ZMAPOR) and in the Ulster Museum, Belfast (BELUM) and are available upon request. The list of studied species and localities with their abbreviations is given in Table S1.

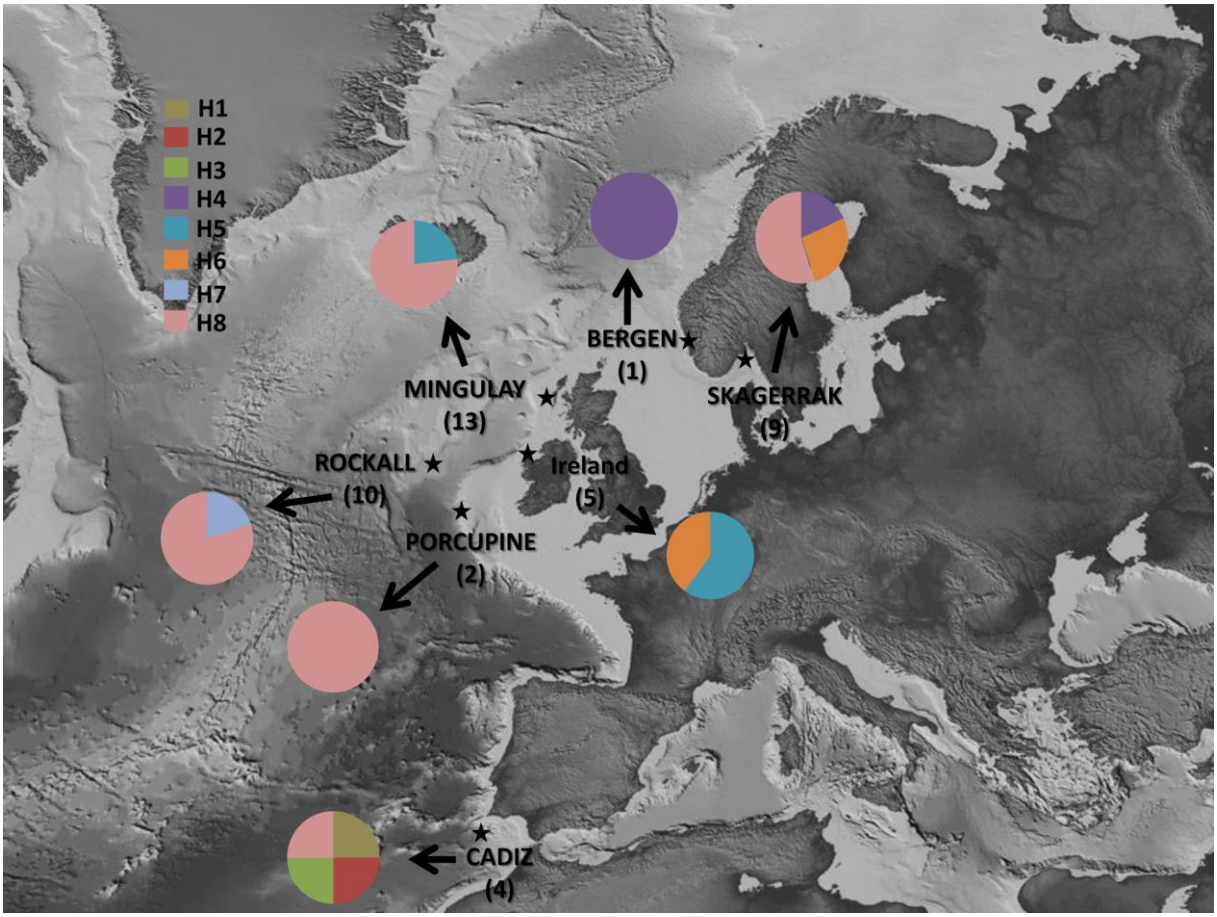

Fig 1. Map showing sampling locations of *Plocamionida* species (numbers in parentheses indicate sample sizes) and geographical distribution of ESUs (H1-H8). Map was provided by the project Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE). Sampling locations are given in uppercase letters for deep-water samples (>50m) and in lowercase letters for shallow-water samples.

DNA extraction, PCR setup and amplifications

DNA extraction from samples was performed using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were carried out in a total volume of 47 µl, with 5 µl of 10 x PCR buffer (Qiagen), 5 µl of 10x CoralLoad (Qiagen), 1 µl $MgCl_2$ (25mM), 1 µl dNTP (10mM each), 0.5 µl of BSA (10µg/µl), 1 µl of forward and reverse primer (25µM), 0.25 µl Top*Taq* DNA polymerase (5u/µl, Qiagen), 1 µl of template DNA and 31.25 µl of HPLC grade water.

A ~1200 bp long fragment of the cytochrome c oxidase subunit I (COI) mtDNA gene was initially amplified from ten random specimens using the universal primer LCO1490 (Folmer et al., 1994) and the reverse primer COX1-R1 (5'-TGTTGRGGGAAAAARGTTAAATT -3') (Rot et al., 2006). Polymerase chain reaction (PCR) cycling conditions included an initial denaturation step of 5 min at 94°C, 5 cycles (94°C for 1 min, 48°C for 1 min and 72°C for 1 min 30), 30 cycles (94°C for 1 min, 50°C for 1 min and 72°C for 1 min 30) and a final extension at 72°C for 10 min. Based on these ten sequences, specific *Plocamionida* sp. primer COIPlo20F (5'-GCTTTTGCGGGGATGATAGGTAC-3') and COI800Rev (5'-TCTACATCCATTCCTACTGTAAACATGTG -3') were developed to amplify the M1M6 partition (Folmer et al., 1994) under a temperature regime of 5 min at 94°C followed by 35 cycles of 94°C for 45s, 47°C for 45s, 72°C for 45s and a final extension at 72°C for 10 min. PCR amplifications of the I3M11 partition were performed using the primers COI800Fwd (5'- CACATGTTTACAGTAGGAATGGATGTAGA-3') (reverse complement from the specific primer COI800Rev) and COX1-R1 under a temperature regime of 94°C for 2 min, followed by 30 cycles of (94°C for 30s, 47°C for 30s, 72°C for 30s) and a final extension at 72°C for 10 min.

The D3-D5 fragment of the rDNA 28S gene fragment was amplified using the primers RD3A 5'-GAC CCG TCT TGA AAC ACG A-3' and RD5B2 5'- ACA CAC TCC TTA GCG GA-3' (McCormack and Kelly, 2002) under a temperature regime described in Reveillaud et al. (2010).

PCR product processing and sequencing

The PCR-amplified products were loaded onto a 1% agarose gel, checked for size, and sequenced in both directions through a Perkin- 234 Elmer ABI 3130 capillary DNA sequencer. The PCR products were purified by incubation at 37°C using exonuclease I, *E. coli* (20 U μl^{-1}; Fermentas) and FastAP thermosensitive alkaline phosphatase (1 U μl^{-1}; Fermentas), and labelled using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Chromatograms obtained from the automated sequencer were read and contigs assembled using the sequence editing software SeqMan Pro v.7.1.0 (DNASTAR Lasergene). We checked the poriferan origin of the sequences by BLAST searches against the Genbank database (http://www.ncbi.nlm.nih.gov/BLAST/) and their relationship to other taxa in a phylogenetic tree as described in Erpenbeck et al. (2002). All the sequences are deposited in the European Molecular Biology Laboratory (EMBL) under accession numbers FR687219-687251.

Sequence alignment and phylogenetic analyses

COI and 28S sequences were aligned using the web interface of the multiple alignment software MAFFT ((Katoh et al., 2002); available at http://www.ebi.ac.uk/Tools/mafft/index.html), under default settings. Ambiguous positions in the D3-D5 region of 28S were discarded. Our own sequences of the poecilosclerid sponges *Desmacella inornata* (Family Desmacellidae) and *Mycale lingua* (Family Mycalidae) were used as uniform outgroup for all analyses.

Both partitions of the COI gene were combined for phylogenetic analyses. The COI and the 28S partitions were separately analyzed and then combined for the same set of specimens whenever possible. A partition homogeneity test performed in PAUP*4.0b10 (Swofford, 2002) with 100 replicates between the COI and the 28S datasets showed that data partitions were not significantly incongruent (p=1). Phylogenetic reconstructions of the nucleotide data sets were performed using the maximum likelihood (ML) criterion of PAUP* 4.0b10 (Swofford, 2002) and Bayesian inference (BI) criterion of MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). We used Modeltest 3.06 (Posada and Crandall, 1998) as well as its simplified version MrModeltest 1.1 (Nylander, 2002) to estimate the best-fitting nucleotide

model under the Akaike Information Criterion (AIC) for each independent gene for the ML and the BI analysis respectively. The GTR+I best fitted the COI data set for both ML and BI reconstructions whereas for the 28S dataset, the models selected by AIC were TrN+I for the ML reconstructions and HKY+I for the BI analyses. The GTR+G+I model was selected for ML and BI for the combined COI and 28S dataset. ML trees were calculated using heuristic searches and a tree bisection and reconnection (TBR) branch swapping algorithm (10 000 rearrangements), and a random stepwise addition of sequences in 100 replicate trials. Nodal support was estimated with a bootstrap procedure with 100 replications and 10 replicate trials of sequence addition. Bootstrap supports (BS) >70 were considered high enough to support clades in ML reconstructions. Bayesian inference analyses were performed with four Markov-chains for each gene. For COI and the combined COI-28S dataset, analyses were performed with 1 million generations sampled every 1000 generations while 300000 generations sampled every 300 generations were used for the 28S dataset. After all analyses, the average standard deviation of split frequencies was below 0.01. We used the burn-in value of 25%. In BI reconstructions, posterior probabilities (PP) >95 were considered to support clades.

Maximum intraclade (whenever more than one haplotype was found) and minimum interclade/branch corrected p-distances were calculated for the COI and the 28S gene fragment using PAUP* 4.0b10 (Swofford, 2002) under the respective models from the ML analyses.

Morphological analyses

Microscopic examination of spicule ornamentations and measurements of spicule micrometries were done using a compound Leitz microscope at 10x10x and 10x40x magnifications, on dissociated spicule mounts obtained after boiling a fragment in concentrated nitric acid, mounted in Canada balsam. All specimens were examined and classified using the following morphological criteria: presence or absence of spines on the blunt ends of the choanosomal large styles, the tylote, mucronate or pointed shape of the tornote endings, the simple or compound shape of the spines in the acanthostrongyles, and size of the chelae (more or less than 30 microns). For the latter character, at least 25 chelae spicules were measured in each preparation and no overlap was found between the ranges 15-25 versus 30+ µm.

Results

Phylogenetic reconstruction

mtDNA COI dataset

The resulting data sets comprised 27 specimens and 12 haplotypes with 777 characters (88 parsimony informative, ts/tv ratio =1.941) for the M1M6 partition and 46 specimens, 13 haplotypes with 363 nucleotides (53 parsimony informative, ts/tv ratio=1.65) for the I3M11 partition (Table S1). Both partitions could unambiguously be aligned and translated into respectively a functional 259 and 121 amino-acid protein sequence of the COI. No frame shifts or point mutations were present. The success rate of the COI amplification in all specimens varied along the COI fragment, with 58 % in the M1M6 partition and 85 % in the I3M11 partition. The combined dataset contained only specimens from which both partitions were obtained. It comprised 27 specimens and 12 haplotypes with 1140 characters (138 parsimony informative, ts/tv ratio =1.685) and was used for the phylogenetic reconstruction of the COI gene (Fig. 2).

Plocamionida specimens formed a well supported monophyletic group, with high Bayesian PP and ML BS (1.0/100). The COI tree was divided into two well supported parts A and B (1.0/100) separated by high genetic divergence values (GTR+I corrected p-distance of 14.5 % to 20 %, Table 1). Part A showed six highly divergent haplotypes (*H1-H6*) with GTR+I corrected p-distance ranging from 1.3 to 5.9 %. *H4* and *H5* were more closely related to each other than to any of the other sequences. In addition *H4-6* and *H2-6* formed well supported subclades (1.0/81 and 0.95/92 respectively). Genetic divergence between the six sequences of Part B was much lower (0.5 % to 1.9 %) than those within part A. Part B was substructured into a well supported clade (*H8a-e*; 1.0/86) and a highly divergent single sequence (*H7*). The sequences *H8d-e* were found to be more closely related to each other than to the other *H8* haplotypes.

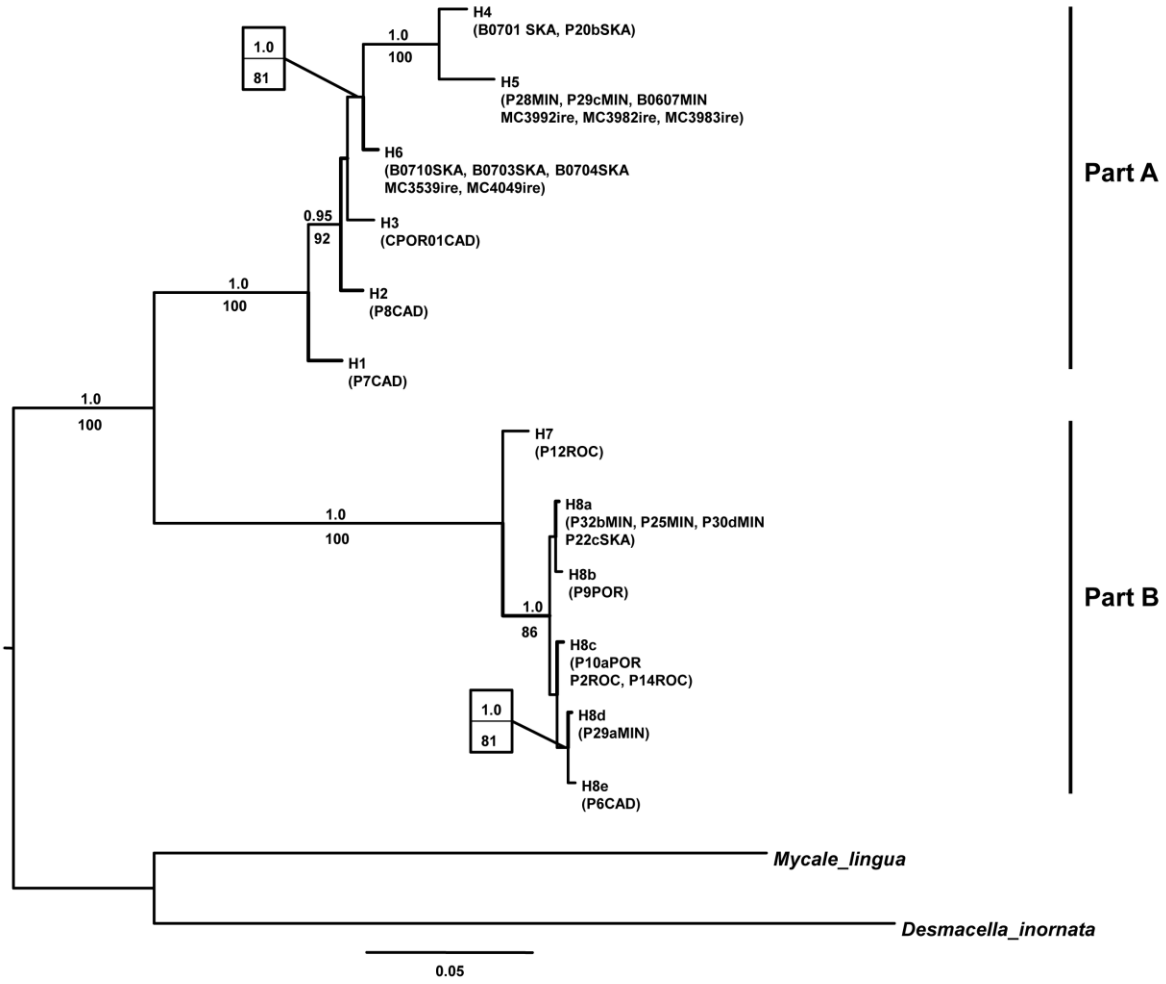

Fig 2. Bayesian majority-rule consensus tree of the mtDNA COI M1M6 and I3M11 partitions. Bayesian posterior probabilities (when > 0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens (listed in parentheses) see Table S1.

Table 1. mtDNA genetic divergence values between and within Evolutionary Significant Unit (ESU)

COI	H7	H8a-e	H4	H5	H6	H1	H2	H3
H7	-							
H8a-e	0,019	0,005						
H4	0,188	0,184	-					
H5	0,197	0,200	0,023	-				
H6	0,165	0,161	0,032	0,040	-			
H1	0,148	0,149	0,055	0,059	0,024	-		
H2	0,147	0,145	0,039	0,042	0,013	0,022	-	
H3	0,155	0,155	0,040	0,045	0,014	0,023	0,014	-

The COI genetic divergence (corrected p-distance) between ESU are provided below diagonal and between individuals within ESU on diagonal. The different haplotypes are presented in Fig. 2.

rDNA 28S dataset

The resulting data sets comprised 39 specimens and 8 genotypes with 620 nucleotides (15 parsimony informative). Phylogenetic relationships using the 28S fragment were highly similar to the ones obtained from COI, but received less support (Fig. 3): *Plocamionida* specimens were recovered as a monophyletic group (-/71) and the deeper parts A and B were recovered (-/85 and 1.0/87, respectively). The subclades *H4-5* and *H4-6* were recovered with high support (0.99/70 and 1.0/79, respectively). The different haplotypes identified in COI as *H8a-e* shared a single 28S sequence (*H8*), with a sequence divergence of 0.1% from *H7* (Table 2).

Sequence divergence ranged from 1.9 to 3.3 % between part A and B, and was 1.2% and 0.1% within Part A and B, respectively (Table 2).

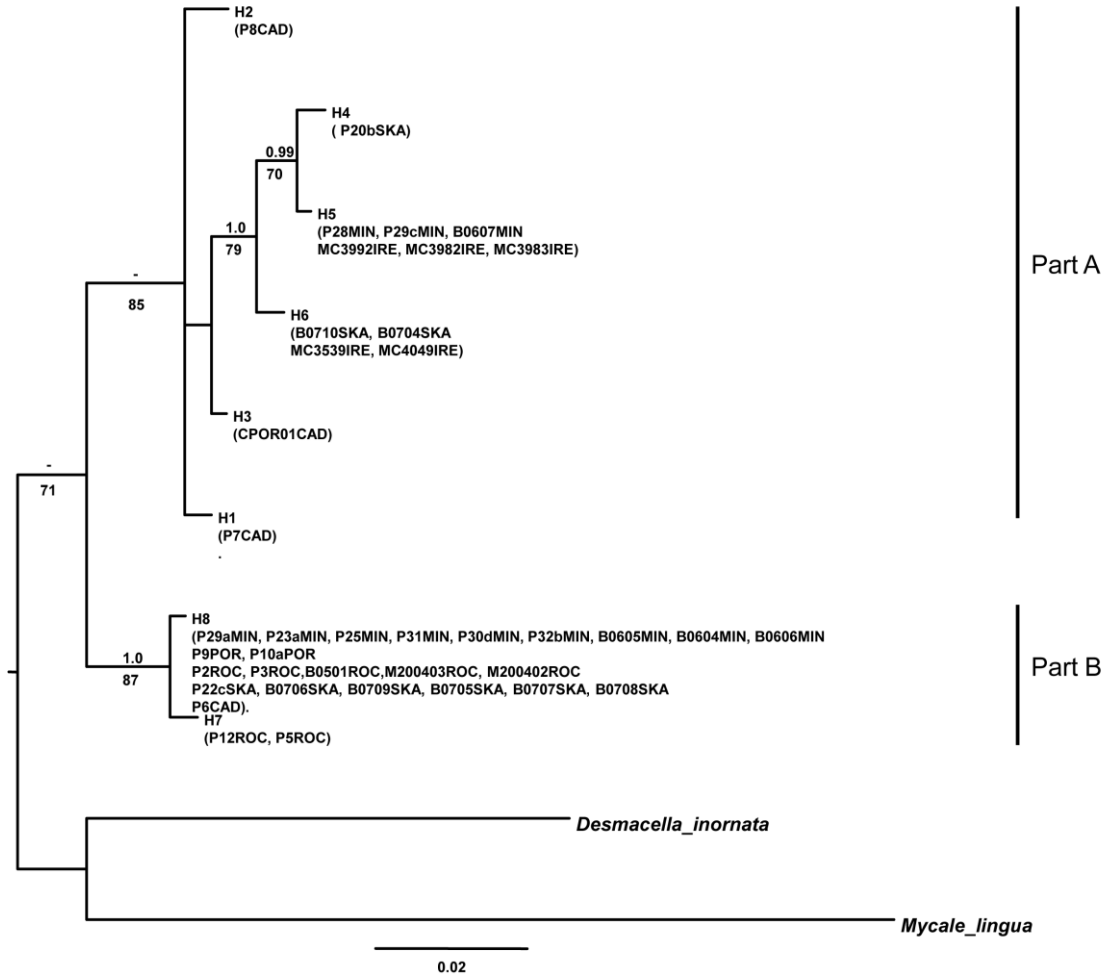

Fig 3. Bayesian majority-rule consensus tree of the rDNA 28S fragment. Bayesian posterior probabilities (when >0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens (listed in parentheses) see Table S1.

Table 2. 28S genetic divergence values between and within Evolutionary Significant Unit (ESU)

28S	H7	H8	H4	H5	H6	H1	H2	H3
H7	-							
H8	0,001	-						
H4	0,033	0,031	-					
H5	0,028	0,026	0,001	-				
H6	0,026	0,024	0,007	0,004	-			
H1	0,020	0,019	0,012	0,010	0,008	-		
H2	0,023	0,021	0,012	0,010	0,010	0,005	-	
H3	0,020	0,019	0,009	0,006	0,005	0,003	0,005	-

The 28S genetic divergence (corrected p-distance) between ESU are provided below diagonal and between individuals within ESU on diagonal. The different haplotypes are presented in Fig. 3

Combined dataset COI-28S

The concatenated COI-28S dataset comprised 24 specimens with 1760 characters. The monophyly of the *Plocamionida* specimens was highly supported (1.0/92), and Parts A and B were again recovered with high support (1.0/94 and 1.0/100, respectively; Fig. 4). *H4* and *H5* were found more closely related to each other as found in COI and subclades *H4-6* and *H2-6* were highly supported (1.0/98 and 0.97/81 respectively). Subclade *H8a-e* within part B was recovered with high support (1.0/96) and *H8d-e* were found to be more closely related to each other, as previously found in COI. Based on the phylogenetic congruence between COI and 28S and the higher genetic divergence value between than within *H1-8* in both genes, we consider hereafter *H1-8* as independent Evolutionarily Significant Units (ESU).

Morphological analysis

Most ESUs were characterized by morphological differences, except *H1-H3* and *H7-H8* (Table 3). Specimens from *H6* were characterized by spined large styles, tylote tornotes, a simple shape of the spines in the acanthostrongyle and the size of the chela being smaller than 30 microns (µm). In contrast, *H4* and *H5* specimens were characterized by a mucronate shape of the tornote endings. The size of the chela further differentiated *H4* and *H5* specimens. As

mentioned above, the three Gulf of Cadiz specimens (*H1*, *H2* and *H3*) were morphologically similar and possessed the tylote tornotes in combination with a large chela > 30 µm. Part B specimens possessed smooth large styles, fusiform tornotes, a truncate shape of the spines in the acanthostrongyle and a chela smaller than 30 µm. The only exception was *H8e* (P6) from the Gulf of Cadiz which had a chela > 30 µm.

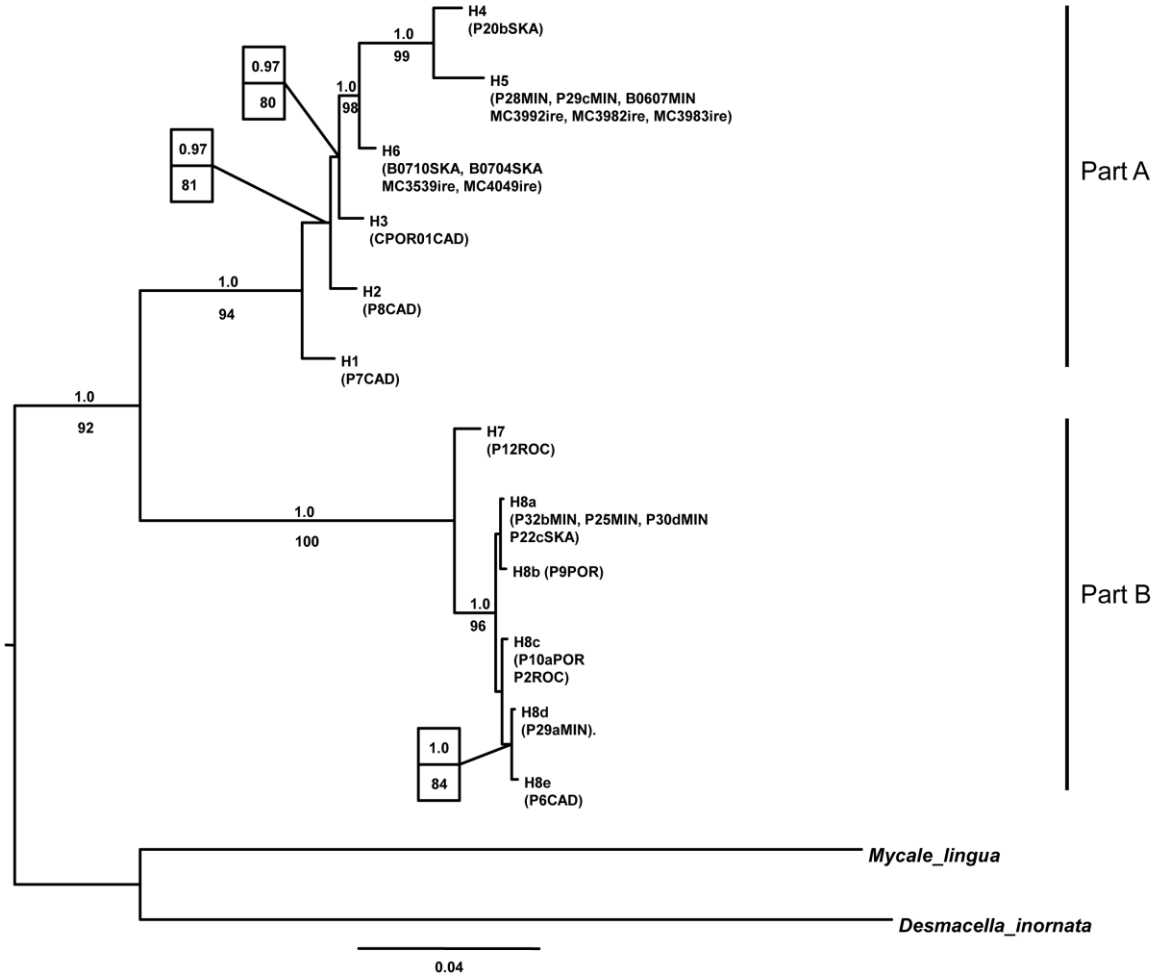

Fig 4. Bayesian majority-rule consensus tree of the concatenated dataset (COI-28S). Bayesian posterior probabilities (when >0.95) and the ML bootstrap values (when >70) are indicated above and below branches, respectively. For information on the specimens (listed in parentheses) see Table S1.

Table 3. Morphological characters of *Plocamionida* specimens.

Sample	*Localities Abb.*	*large style*	*tornotes*	*acanthostrongyle*	*chela*	*Identity*
M2004-02	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
M2004-03	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P2	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P3	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P4	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P11	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P14	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B05-01	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P9	**POR**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P10 a	**POR**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P23 a	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P25	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P29a	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P30d	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P31	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P32b	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B06-03	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B06-04	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B06-05	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B06-06	**MIN**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B07-05	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B07-06	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B07-07	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B07-08	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
B07-09	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P22c	**SKA**	**smooth**	**fusiform**	**truncate**	**<30**	**H8**
P6	**CAD**	**smooth**	**fusiform**	**truncate**	**>30**	**H8**
P5	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H7**
P12	**ROC**	**smooth**	**fusiform**	**truncate**	**<30**	**H7**
B07-03	SKA	spined	tylote	simple shape	<30	H6
B07-04	SKA	spined	tylote	simple shape	<30	H6
B07-10	SKA	spined	tylote	simple shape	<30	H6
MC3539	ire	spined	tylote	simple shape	<30	H6
MC4049	ire	spined	tylote	simple shape	<30	H6
P28	MIN	spined	mucronate	simple shape	<30	H5
P29c	MIN	spined	mucronate	simple shape	<30	H5
B06-07	MIN	spined	mucronate	simple shape	<30	H5
MC3992	ire	spined	mucronate	simple shape	<30	H5
MC3982	ire	spined	mucronate	simple shape	<30	H5
MC3983	ire	spined	mucronate	simple shape	<30	H5
P20b	SKA	spined	mucronate	simple shape	>30	H4
B07-01	SKA	spined	mucronate	simple shape	> 30	H4
BER82-01	BER	spined	mucronate	simple shape	> 30	H4
CPOR08-01	CAD	spined	tylote	simple shape	> 30	H3
P8	CAD	spined	tylote	simple shape	> 30	H2
P7	CAD	spined	tylote	simple shape	> 30	H1

Specimens, their localities abbreviation (Abb.) as in Table S1, examined for the following morphological criteria: presence or absence of spines on the blunt ends of the choanosomal large styles, the tylote, mucronate or pointed shape of the tornote endings, the simple or compound shape of the spines in the acanthostrongyles, and size of the chelae (more or less than 30 microns). Their identity is provided by their corresponding Evolutionary Significant Unit (ESU). Part B's specimens in bold.

CHAPTER V

Discussion

Integrative Taxonomy in Plocamionida

The genetic results conform well to previous morphological proposals for the subdivision of *Plocamionida* into several distinct European taxa. The specimens are divided into eight ESUs, which were grouped into two clades: Part A (*H1-6*) and part B (*H7-8*) specimens were separated by high divergence values in the COI and 28S fragments (minimum corrected p-distance of 14.5 % and 1.9 % respectively) and showed consistent morphological differences (spined large styles in Part A specimens vs. smooth large styles in Part B specimens). Comparing the limited number of morphological characters against a robust and comprehensive phylogenetic (DNA) tree proved to be a fruitful approach for integrating the strengths of morphological data with those of sequence data.

Part A contains *Plocamionida ambigua* s.l. and the forms described by Brøndsted (1932) as *f. tornotata*, *f. tylotata* and *f. grandichelata.* Specimens of ESU *H5* possess the characters described by Bowerbank (1866) for the type specimen of *Plocamionida ambigua* and by Brøndsted (1932) as *f. tornotata*. Haplotype *H6* specimens possess the characters described by Brøndsted (Brøndsted, 1932) as *f. tylotata* and haplotype *H4* specimens possess the characters described by Brøndsted (Brøndsted, 1932) as *f. grandichelata*. Given these morphological differences and the high genetic distance between *H4* and *H6* specimens (corrected p-distance of 3.2 % in COI and 0.7 % in 28S), as well as between *H4* and *H5* (corrected p-distance of 2.3 % in COI and 0.1 % in 28S), *f. tylotata* and *f. grandichelata* can be considered valid species, whereas *f. tornata* corresponds to the species *P. ambigua*. Three Gulf of Cadiz specimens of Part A (*H1*-3) appear to have deviating characters from the other *Plocamionida* specimens (spined large styles, tylote tornotes, simple shape of the spines in the acanthostrongyle and large chelae). This observation, in addition to the presence of unique haplotypes, divergent from the most closely related species (*P. tylotata, H6*) by p-distance values from 1.3 % to 2.4 % in COI and from 0.5 % to 1 % in 28S suggests that these specimens may actually be undescribed *Plocamionida* species. However, no morphological characters could distinguish the three different specimens from each other. Evidently, a larger number of specimens from these ESUs need to be analyzed to infer whether they may form a cryptic species complex.

Part B conforms almost entirely with the description of *Plocamionida microcionides* (Carter, 1876) as redescribed by Stephens (1921). Only the above mentioned H8e from the Gulf of Cadiz deviates by having large chelae, a characteristic used so far as critical to delineate species, in combination with molecular data. This one COI sequence indicates that the classification of species purely by means of morphology may be difficult and that the size of the chelae within *Plocamionida* species can be an ambiguous diagnostic character in some *Plocamionida* species. It again emphasizes the need to study morphological variation in combination with other data, such as genetic variation. In addition, *H7* is highly divergent from the other *P. microcionides* specimens in the molecular analyses of COI and 28S (p-distance values of 1.9 % and 0.1 % respectively) and the combined partitions, but its morphological features are identical to the ones of Part B. This may indicate that *P. microcionides* is actually a species complex. However, more specimens of *H7* are required to support this hypothesis. Additional investigations (behavioural, ecological, etc.) and further taxonomic analyses (cytology, chemistry) might also be needed.

The high and non-overlapping genetic divergence values among ESUs (from 1.3 % to 20 %), and within the more widespread and genetically diverse species *H8* (0.5 %, Table 1) indicates the usefulness of the COI partitions for the molecular distinction of species in our data set. A slightly lower ratio of transition/transversions (1.45 vs. 1.941) was observed in I3M11 vs. M1M6 partitions when using the 27 specimens for which both M1M6 and I3M11 sequences were available. It confirms the more progressive stage in character evolution of the I3M11 partition compared to the M1M6 partition (Erpenbeck et al., 2006b). Moreover, the I3M11 partition was much easier to amplify than the M1M6 partition. Sequences of the M1M6 partition were more often impeded by contaminations (hydrozoans, microbial symbionts, etc.). Our study further confirms the resolution power and suitability of the I3M11 COI partition for low level phylogenies such as barcoding, (Sponge Barcoding project, http://www.spongebarcoding.org/), as the same number of ESU (eight) was detected using the M1M6 and I3M11 partitions separately or even jointly. Although the low COI genetic differences between some of the ESUs (Table 1:1.3 to 1.9 %) are clearly smaller than the interspecific distances found within other genera, such as *Hexadella* (Order Verongida, 3.9 to 8.7 %; Reveillaud et al., 2010) or *Scopalina* (Order Halichondrida, 11 to 22 %; Blanquer and Uriz, 2007), Poppe et al. (2010) reported very low genetic distance values (maximum 1.8 %) between morphologically distinct *Psammocinia* species (Order Dictyoceratida).

Consequently, the COI marker seems to show different levels of genetic variation between different sponge taxa. The 28S tree showed major congruences with the COI tree, although the number of highly supported clades recovered in 28S was lower. This marker, in combination with COI, was found suitable to highlight putative cryptic species within *Plocamionida,* such as *H1-3* and *H7-8*. The combination between molecular data and morphological characters proved useful for differentiating *Plocamionida* species and for establishing their phylogenetic relationships. All trees showed the same topology, which confirms the consistency of the arrangement. This study reinforces the utility of integrative taxonomy (Dayrat, 2005; Wahlberg et al., 2005; Blanquer and Uriz, 2007; Fonseca et al., 2008).

Bathymetric and geographic distribution of Plocamionida species

P.tylotata, and *P.ambigua* were shown to present a wide bathymetric range distribution, with records in both deep (Skagerrak and Mingulay CWRs respectively), and shallow water (the Irish locality), while *P.microcionides* and *P.grandichelata* were found only in deeper habitats (>= 50 m; Carter, 1876; Stephens, 1921; Brøndsted, 1932; this study). So far, *P. grandichelata* was only reported from Scandinavian waters (the Faroe, Brøndsted, 1932); CWRs area from Bergen and Skagerrak, this study). Interestingly, our data suggests that the deep habitats of the Gulf of Cadiz area harbors the highest diversity, with four ESUs observed out of four samples (Fig. 1). Currently there is one described *Plocamionida* species from the Antarctic (*Plocamionida gaussiana* (Hentschel, 1914)), one from Washington (USA) (*Plocamionida lyoni* (Bakus, 1966)), one from St Georges, Grenada (*Plocamionida topsenti* Burton, 1954) and none of them have been reported in the NE Atlantic. In addition, *P. gaussiana* and *P. lyoni* may lack proper short echinating acanthostyles, a morphological criterion shared by *P. topsenti* and the European *Plocamionida* species. Consequently, most of the ESUs investigated here might represent new taxa and our data show that the current number of *Plocamionida* species of the NE Atlantic waters may be underestimated. We did not encounter any individual that could be assigned to an *achelata* variety Topsent, 1928, but its existence may also indicate further diversity in the genus *Plocamionida*. A possible radiation of *Plocamionida* in the Gulf of Cadiz is suggested by our data, supporting the idea that 'low latitude' CWRs act as diversity hot-spots. Similarly, the bathyal Gulf of Cadiz area showed particularly high species diversity of marine hydroids (Moura et al., 2008), while an

unprecedented number of unique evolutionary lineages of tubeworms was reported from the Gulf of Cadiz mud volcanoes (Hilario et al., 2010). Obviously, the sharp environmental discontinuities in temperature, pressure and nutrient richness (including silica concentration) in shallow water vs. deep-water coral reef habitats may have a great potential for sponge evolution. Our study adds to the growing evidence of genetically highly diverse CWRs and is expected to contribute to an improved understanding of the role of CWRs in the sustenance of sponge distributions along the coasts of Europe.

Conclusion

Following an "integrative taxonomy" approach to study species from multiple, complementary perspectives (Dayrat, 2005), this study provides evidence that *P. grandichelata*, *P. tylotata*, *P. ambigua* and *P. microcionides* in the NE Atlantic are valid species, and suggests the existence of putatively new *Plocamionida* species. Those hypothetical species are now submitted to the filter of other approaches and further sampling followed by detailed phenotypic diagnostic analyses may support the observed molecular differences. New species are indeed important to consider for the protection of cold-water coral reefs, which are increasingly shown as reservoirs of biodiversity. On the other hand, the unexpected high level of *Plocamionida* biodiversity, found in the Gulf of Cadiz especially illustrates the problem of obtaining sufficient specimens in any one deep-sea sponge species from CWRs for phylogeographic studies. The higher amplification success and higher resolution power of I3M11 adds to the growing evidence that it may be a better COI partition than M1M6 to infer inter- and intraspecific diversity.

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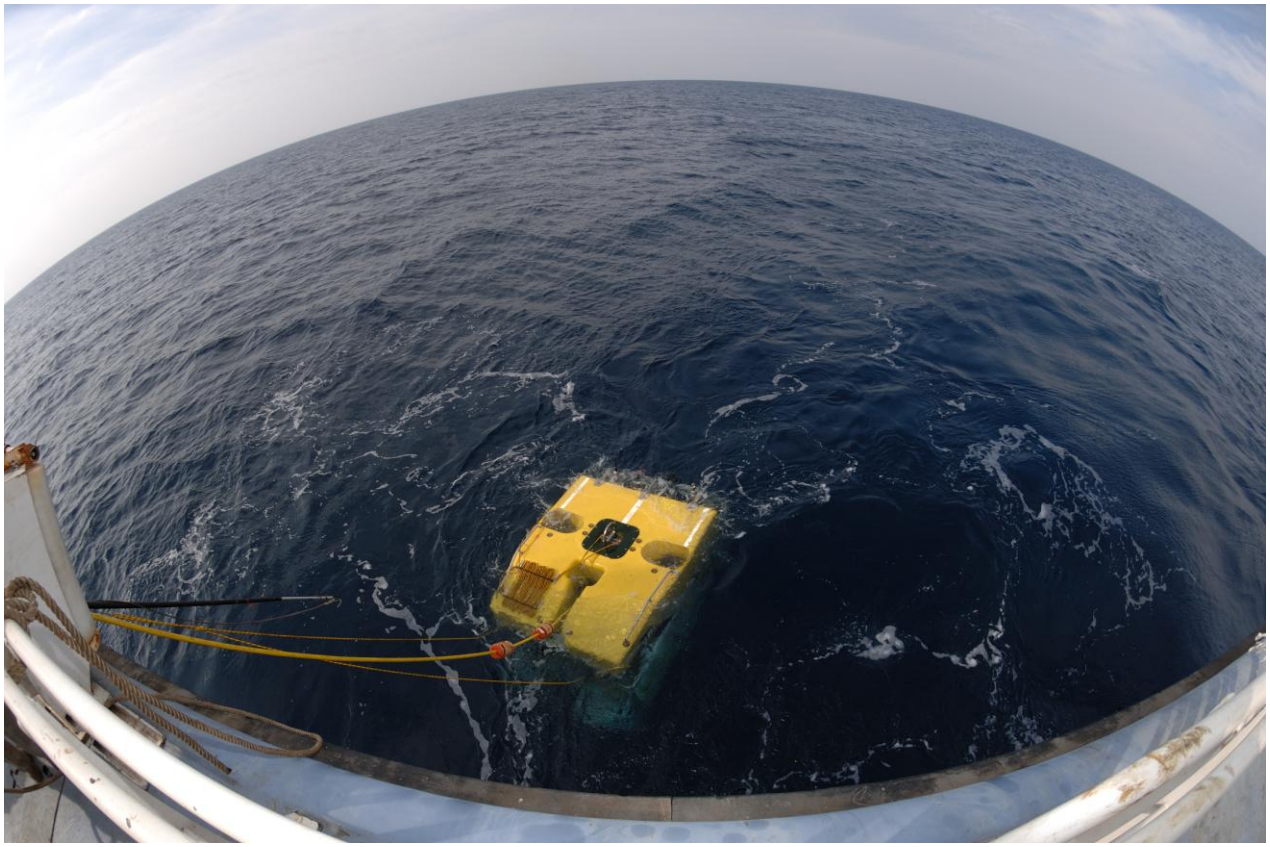
Author contributions

Conceived and designed the experiments: JR RS. Performed the experiments: JR AR RS. Analyzed the data: JR RS BP SD. Contributed reagents/materials/ analysis tools: AV RS. Wrote the paper: JR RS SD.

CHAPTER VI

General Discussion



In the present thesis, we focused on species delineation in sponges occuring from shallow-water to deep-sea coral ecosystems in the Mediterranean Sea and along the North East Atlantic margin. We investigated the phylogenetic relationships within small encrusting sponges from the genus *Hexadella* and *Plocamionida* using independent molecular markers. These data were combined with additional information (e.g., morphological, cytological, biochemical, toxicity data) to provide an integrative approach to sponge species identification. In this last chapter, we discuss patterns of cryptic species, genetic endemism and connectivity of reefs along the European margins. In an attempt to understand phylogenetic diversification of sponges over evolutionary time scales, we also identified possible links between the observed patterns of diversity and the processes influencing the diversity of benthic communities in the deep sea. Furthermore, the resolution, advantage and drawbacks of the different markers used in this study are addressed. We also seize the opportunity to discuss, in light of the data presented in this thesis, how the combination of independent sources of information can provide relevant tools for the identification and description of some problematic sponge taxa. This thesis finally also illustrates that the study of species limits provides essential information for the conservation and sustainable management of an ecologically, biochemically and economically important group of organisms.

Are cold-water coral ecosystems hotspots of biodiversity for sponges?

Species boundaries analyses conducted in this thesis allowed the identification of previously unrecognized units of biodiversity in the phylum Porifera. These phylogenetic studies illustrated the evolutionary distinctiveness of several lineages within the genus *Hexadella* in **both shallow-water and deep-sea environments**. Before this study, three species of *Hexadella* were described from the Northeast Atlantic and Mediterranean Sea: the deep-sea species *H. dedritifera*, and the shallow-water taxa *H. pruvoti and H. racovitzai*. Our phylogenetic analyses, based on the congruence of independent nuclear and mitochondrial markers have suggested potentially new deep-sea cryptic species *H. cf. dedritifera* (maybe a junior synonym of *H. pruvoti*), and *Hexadella* sp. (at the moment only known from a deep-sea coral bank in the Ionian Sea) as well as shallow-water species *H. topsenti* and *H. crypta* (see their formal description in CHAPTER IV). Similarly the phylogenetic study on *Plocamionida* suggested an underestimation of the current number of *Plocamionida* species in **the deep-sea** NE Atlantic waters. The combination of morphological and genetic analyses on *Plocamionida* provided further evidence that the 'formas' *Plocamionida ambigua f. tylotata* and *f.*

grandichelata can be considered valid species while *P. ambigua f. tornata* corresponds to the species *P. ambigua* sensu stricto. In addition, the deep-sea *P. microcionides* was shown as **really divergent** from the other taxa and four putative new **deep-sea** *Plocamionida* species (H1, H2, H3, H7) were suggested by our data. These findings are in accordance with the low dispersal potential attributed to Porifera (i.e., sponges are known to disperse by means of lecitotrophic larvae and display philopatric behavior; Mariani et al., 2005, 2006; Uriz et al., 1998), and adds to the growing number of cryptic species reported in Porifera. In addition, these results stress a particularly high diversity of sponges in deep-sea coral ecosystems: possibly six new small encrusting sponge species (from the genus *Hexadella* and *Plocamionida*) were recorded from deep-sea environments, while only two new shallow-water *Hexadella* species are reported. First, such patterns of high interspecific variation in sponges from deep-sea coral ecosystems are in accordance with the overall high diversity levels reported in the deep sea (Grassle and Macioleck, 1992; Ramirez-Llodra et al., 2010). Moreover, it illustrates that the complicated taxonomy of small sized and morphologically cryptic sponges, which require high resolution microscopy and expert knowledge (Van Soest et al., 2007a), is even amplified in the deep sea where great species diversity is reported.

In addition, our results show a notably **high genetic diversity** within sponge species from deep-sea coral ecosystems. Seven distinct COI haplotypes corresponding to three possible deep-sea *Hexadella* species were observed out of 33 deep-sea samples, whereas only four haplotypes corresponding to four shallow-water *Hexadella* species were found in 40 shallow-water samples (CHAPTER III AND IV). Likewise we reported 12 divergent *Plocamionida* COI haplotypes in the deep sea, whereas only two were found in the shallow-water environment. Although the study included only six shallow-water samples versus 22 deep-sea ones, it is noteworthy that four divergent deep-sea *Plocamionida* lineages, including three putative new taxa, were reported out of four samples from a single location (e.g., the bathyal environments of the Gulf of Cadiz). This pattern of high genetic diversity in sponges from deep-sea coral ecosystems and the contrasting lack of genetic variation within sponge species from shallow-water environments (e.g., *H. pruvoti*, *H. racovitzai*, *H. topsenti*, *H. crypta)* provide further evidence for the high diversity of deep-sea habitats. Although the diversity and ecology of deep-water coral habitats is still poorly investigated, it was suggested that these upper bathyal coral ecosystems are highly dynamic environments which provide opportunities for the evolution and diversification of taxa that subsequently invade other

regions (Lindner et al., 2008). Despite it has long been proposed that deep-water fauna originated from shallow-water ancestors, Lindner et al. (2008) demonstrated the diversification of corals in the deep sea followed by their invasion in shallow water.

Genetic endemism

These results demonstrate for the first time the presence **of cryptic, deeply divergent lineages** in a major group of benthic marine invertebrates associated with deep-water coral ecosystems along the European margin, and increase our awareness of the biodiversity of sponges in the deep sea. Because of its remoteness, sponge diversity studies in deep-sea coral ecosystems have not been addressed very thoroughly until recently. Although species diversity studies of sponges in cold-water coral (CWC) ecosystems reveal high species richness and heterogeneity (Van Soest and Lavaleye, 2005; Van Soest et al., 2007a), no species was found endemic to coral reefs. When comparing sponge species composition on and off reefs, the habitats surrounding the reefs contained an impoverished 'reef' fauna instead of a separate habitat for Porifera. Similarly here, *Hexadella* was found on coral branches but also overgrowing stones or other sponges (personal observation). Nevertheless, the lack of endemism in sponge species associated with coral reefs should be treated with caution because sponges were identified by morphology alone. This study indeed adds to the growing evidence that marine organisms with a paucity of morphological characters, such as Porifera, hide substantial genetic differences, even across regions without evident barriers to gene flow. Our results show that although some lineages are widespread, they are geographically more restricted than previously thought. Some taxa have highly restricted ranges (e.g., *P. grandichelata* was only reported from deep-habitats in Scandinavian waters), making them vulnerable to extinction. In addition, some new-to-science species were found to cluster into specific deep-sea habitats. Our work on *Plocamionida* highlighted phylogenetic lineages of sponges so far exclusively found in the Gulf of Cadiz, suggesting that this region is **a center of genetic endemism**. Our data indeed suggested a possible radiation of *Plocamionida* in this area, and therefore supported the idea that **"low latitude" CWC reefs act as diversity hot-spots.** Although a more detailed sampling would allow more definitive conclusions (cf section on Future directions), these results are concordant with previous studies on other organisms such as the Hydrozoa, in which the bathyal Gulf of Cadiz area showed particularly high species diversity (Moura et al., 2008) or in Polychaeta, where an unprecedented number of unique evolutionary lineages of Siboglinid tubeworms was reported

from mud volcanoes (Hilario et al., 2010). Although the Gulf of Cadiz currently appears as an area with a high occurrence of mainly dead corals, it is noteworthy that it holds a great diversity of marine organisms. Next to the Gulf of Cadiz, the deep-sea reefs in the Ionian Sea harboured three unique specimens (*Hexadella* sp) showing a color variation when fixed in ethanol (dark green instead of dark violet). Their sequence (Seq1, see CHAPTER III) had a basal position to all other *Hexadella* specimens and was found to significantly diverge from the sympatric *H. cf. dedritifera* (genetic distance of 4.2 % in the COI fragment and of 14.7 % in the ATPS fragment). These data add to the growing evidence of genetically highly diverse CWC reefs in Southern Europe and are expected to contribute to an improved understanding of the role of CWC ecosystems in the sustenance of sponge distributions along the coasts of Europe.

Are cold-water coral ecosystems connected or isolated along the European Margins?

Considering that sponge larvae were shown to be recruited at short distance from the parental locations (Mariani et al., 2005, 2006; Maldonado 2006; Uriz et al., 1998), sponges are assumed to have limited dispersal abilities. In addition, some marine population studies emphasize that local oceanographic processes and the timing of spawning events are likely to limit larval dispersal by increasing local retention (Cowen et al., 2006), which can produce demographically discrete populations. Therefore, populations are expected to be structured in most sponge species. Studies on sponge phylogeography and population genetics in the Northeast Atlantic and the Mediterranean Sea are scarce (Duran et al., 2004; Blanquer et al., 2009; Xavier et al., 2010b), but worldwide the available data shows highly structured shallow-water populations as a result of restricted gene flow at various spatial scales (e.g. Wörheide et al., 2002; Wörheide, 2006; Bentlage and Wörheide, 2007; Wörheide et al., 2008; López-Legentil and Pawlik, 2009). Nevertheless, our results on deep-sea populations of the newly identified *Hexadella* species show signatures of **gene flow across large areas**, and therefore suggest a complex evolutionary history of deep-sea sponges along the European margins. Findings of high genetic similarities between specimens from the Ionian Sea, the Bay of Biscay, and the Irish margin (*H. cf. dedritifera,* yellow arrows, Fig.1) and between specimens from the Irish, the Scottish, the Norwegian margins and the Greenland Sea (*H. dedritifera* sensu strict, red arrows Fig 1) suggest a past gene flow between deep-sea reefs. The lack of COI genetic divergence across *Hexadella* specimens from reefs separated by thousands of kilometers could be due to the slow evolution of the mitochondrial marker, as

already shown in other sponge species taxa (Duran et al., 2004; Wörheide, 2006); however, very low genetic divergence in the highly variable ATPS marker supported this gene flow hypothesis (see discussion below on the different resolution of markers in sponges).

These first genetic results on deep-water coral associated sponges are in accordance with the moderate genetic differentiation and sporadic gene flow reported among the dominant coral *Lophelia pertusa* offshore subpopulations along the European margin (Le Goff-Vitry et al., 2004; Le Goff-Vitry and Rogers, 2005). It therefore adds to the growing evidence of a **certain genetic cohesion among reef populations along the European margins**.

Overall, the pattern of genetic similarity found here, appears to agree with main ocean currents along the slope. Ocean currents, such as the Mediterranean Outflow Water (MOW) were suggested to act as colonization drivers of cold-water corals and associated fauna towards the North (De Mol et al., 2005; Roberts et al., 2006), while patches of CWC reefs may represent stepping-stone habitats for larval dispersal along the North East Atlantic margin (Reveillaud et al., 2008, see below). Lopez-Legentil and Pawlik (2009) suggested that the pattern of genetic structure within the sponge species *Xestospongia muta* is related to patterns of ocean currents in the Caribbean. In 2010, Xavier et al. also found evidence of long-distance dispersal events between some populations of the shallow-water sponge *Phorbas fictitius,* and illustrated the importance of current patterns in a climate-changing environment. In addition, the results of the present study query the possible unusual life history traits of these sponge species. Actually, it has been recently shown in laboratory conditions (Maldonado, 2009) that larvae from the order Verongida (*Aplysina aerophoba*) can swim for 7 days before settling, indicating a possible great potential for dispersal in some aspiculate demosponges.

Fig. 1: Genetic differentiation corresponding to the different *Hexadella* cryptic species (illustrated by the different colours) and connections (showed by arrows) suggested between deep-water sponge populations associated with coral reefs along the European margins.

In the deep sea, which is a food-limited environment compared to shallow water (Gage and Tyler, 1991), the connection among sponge populations across large distances (some thousands of kilometers) also raises questions about the possibly slower developmental rates and longer planktonic period of some sponge larvae. This aspect is crucial and needs further investigation in the near future.

The lack of differentiation between the three Northern populations of *P. microcionides* (26 individuals) may also support a certain genetic cohesion among deep-sea sponge populations (ADDENDUM III). The fact that individuals from the Norwegian margin grouped with individuals from the Irish and Scottish margin could indeed be a result of the colonization of the Skagerrak area by migrants originating from southern populations.

However, although our *Plocamionida microcionides* dataset could be suitable to draw some tentative conclusions about phylogeography and connectivity, a more extensive sampling is necessary to firmly support these assumptions. In addition, other more variable markers, such as microsatellites or SNPs (Single Nucleotide Polymorphism), should be studied to confirm this pattern. The lack of significant genetic structure within *P. microcionides* from the Irish to the Norwegian margin could indeed reflect a lack of statistical resolution, due to the limited number of individuals. But it could also be caused by the random evolutionary history of the locus analyzed, as selection and incomplete lineage sorting can affect patterns of genetic differentiation (Whitlock and McCauley, 1999). Nevertheless, this *Plocamionida* dataset (i.e., the high diversity of lineages reported in the deep sea) exemplifies the **difficulties to obtain a sufficient number of individuals from a deep-sea species** in order to perform a phylogeographic/population genetic study of organisms associated with cold-water corals. This study therefore highlights the limits of deep-sea sampling programs for genetic diversity studies and suggests for future investigations to design cruises dedicated to this research. Instead of sharing on-board-time between different experts (e.g., geologists, biologists, physical oceanographers, etc), we suggest the sampling of species from different phyla across the same areas for comparative phylogeographic studies (see section below on suggestions for future research). The condition is of course that we know the environment well.

Are the deep-sea continental margins providing a continuous habitat for reef colonization?

The continental margin is actually suggested to act as a biogeographic area, where extreme populations are connected through intermediate, stepping stone populations and where isolation by distance effects may be weak (Palumbi, 1994). The very low genetic divergence values across *Hexadella* populations from reefs thousands of kilometers apart (Ionian Sea-Rockall Bank, 5200kms) suggest intermediate, **unsampled locations playing a stepping stone role** along the European margins. The connection could indeed be the result of sporadic gene flow between intermediate (unsampled) localities. These data further support our compilation of scattered scleractinian reports from the Bay of Biscay (CHAPTER II), which highlighted **key regions of coral reefs clusters along the slope** (e.g., the Meriadzek Terrace, the Banc de la Chapelle, the Aquitaine margin, the Cap Breton Canyon, Le Danois Bank and the Cantabrian–Galician margin). These areas were suggested to constitute stepping stone key populations for larval dispersal and therefore, the Bay of Biscay was suggested as a

semi-continuous habitat for cold-water coral occurrences. Actually, in a worldwide biogeographic study of scleractinian corals, Cairns and Chapman (2001) highlighted that one of the largest biogeographic clusters extended from Great Britain to Senegal, showing also some affinities with the Mediterranean Sea in the east and the Faroe Islands in the North. As such, the Bay of Biscay showed a **central position within the European margin,** and represented a crucial and obligate transit route for coral colonization between the southern and northern reefs from the European margin (CHAPTER II).

This key-stone sector of the NE Atlantic continental margin represented a case study for a better understanding of the distribution of coral and associated fauna along the European margins and the factors that regulate them. Mapping the distribution of corals along the margin in the Bay of Biscay highlighted (i) that a remote and steep slope, difficult to sample, and with inaccessible topography may provide an indirect protection for coral reefs; (ii) that an area **characterized by strong bottom currents and water mixing** may support the development of filter feeder/suspension feeder colonies and may imply random disturbance of benthic communities; (iii) that there is a close association of **dead and live scleractinian corals in local areas**.

Investigations directed to a better understanding of cold-water coral ecosystems in Europe first focused on live corals. In the Bay of Biscay, historical studies mentioned large patches of live corals (Joubin 1922, Le Danois 1948). However, boxcores from recent cruises in the same area were shown to contain a large amount of dead fragments or coral rubble with only a few living corals present (e.g., *Dendrophyllia cornigera)*. Therefore**,** this regional study (Chapter II) in the Bay of Biscay stressed that both live and dead coral occurrences may be highly frequent along the European margins. Actually, dead branches and living corals were found to co-occur in the NE Atlantic (e.g., on the Faroe shelf: Jensen and Frederiksen, 1992; along the Irish margin: Huvenne et al., 2005; Foubert et al., 2005; De Mol et al., 2002; 2005; in the Bay of Biscay: Reveillaud et al., 2008, this thesis), while coral graveyards dominate some other areas, such as in the Gulf of Cadiz along the Moroccan margin (Wienberg et al., 2009). Nevertheless, coral rubble and dead fragments can serve as hard substratum for the initial settlement of larva, and may therefore be important for any putative recolonization. In contrast, disturbance of coral rubbles may prevent the preservation of an area free of resuspended sediment and may even impede polyp respiration. Genetic studies highlighted

very low genetic differentiation of *Lophelia pertusa* in disturbed and stressed areas (Le Goff-Vitry et al., 2004) and in regions of intensive trawling, where so far no reproductively active polyps were found (Waller and Tyler 2005). The lack of genetic structure of sponges and the distribution of corals along the Bay of Biscay margin suggest that both dead and live coral skeleton, providing hard substrate, may play a critical role for settlement and therefore colonization by larvae from neighboring isolated coral reefs, in the Bay of Biscay and possibly in other areas along the margins. We therefore suggest to include both live and dead coral occurrences from an area in any conservation efforts on cold-water coral ecosystems.

Why are cold-water coral ecosystems so diverse?

Phylogeography aims at addressing large-scale evolutionary patterns related to biogeographical barriers. In such a context, the question is which biogeographical barriers influence diversification in the deep sea, and especially in deep-sea coral reefs. Isolation by distance followed by posterior colonization of the species is often the most accepted mechanism of diversification. This is the **model of allopatric speciation** or speciation due to geographic isolation (Mayr, 1963), which is occurring in shallow-water coral reefs (see Rocha et al., 2007). Actually, Pleistocene (2,588,000 to 12,000 years BP) diversification from refugia has been suggested to play a diversity role in the marine realm (Palumbi, 1994). Indeed the long-term isolation of populations within geographically separated refugia during the numerous quaternary glaciations is suggested to have promoted genetic divergence between populations (shifts in allele frequency) and species (Rocha et al., 2007). A current model of glacial refugia (Hewitt, 2000) supports *genetically more diverse* populations in the South and a lower diversity in locations that suffered the strongest anomalies during the glaciations (Maggs et al., 2008). As such, refugia might be differentiated from admixture zones (Provan and Bennett, 2008) because the latter will be composed entirely of haplotypes from the source refugia (or their descendants), whereas the refugia themselves tend to harbour high numbers of private haplotypes found in no other populations. These haplotypes, which may be represented by a very small number of individuals, might get lost by genetic drift after participating in the recolonization process. Measuring divergence between haplotypes can also be a useful indicator for the regional status of refugia as *haplotypes within a refugium will be more closely related* genealogically to haplotypes from the same refugium than to those from other refugia.

Although discrepancies between patterns of genetic and species diversity have been widely noted previously (Fjeldsa et al., 1994), in the present case study molecular and morphological data support the idea that the southern NE Atlantic cold-water coral reefs are areas with high diversity. Despite the small sampling size, the *high number of restricted Plocamionida* ESUs separated by relatively low genetic divergence (1.3 % to 2.3 %) found in the Gulf of Cadiz support the hypothesis of refugia and diversification centers within the low latitude coral reefs (Roberts et al., 2006). All other deep-sea *Plocamionida* were widespread along the European margins (i.e., ESUs H4-H5-H6 and H8), except ESU H7 which was found exclusively in the Rockall bank. Such restricted lineage could highlight a northern refugium in the area, but obviously a greater sampling scheme is needed to support such hypothesis. During glacial periods, southward faunal shifts of deep assemblages from the North East Atlantic to lower latitudes have actually been suggested (Taviani et al., 1991). Although our provisional phylogeographic analysis needs to be confirmed by additional samples, the observed higher genetic diversity at lower latitudes combined with the geographical distribution of COI haplotypes within the species *P. microcionides* (ADDENDUM III) suggest a northward post-glacial expansion from low to high latitude CWC reefs in the NE Atlantic. This genetic pattern is also consistent with paleoclimate evidences for the Holocene age of exposed occurrences of deep-water corals in Scandinavian waters, the Faroe area and the Rockall Trough, while deep-water corals from lower latitudes off NW-Africa, the Mid-Atlantic Ridge zone and western Mediterranean Sea (i.e., the strait of Gibraltar) show continuous growth over the last 53.5 ka (Schroder-Ritzrau et al., 2005; Wienberg et al., 2009).

Reef growth appears to be strongly related to the environmental forcing of the major oceanic circulation pattern (Ruggeberg et al., 2005). Colin et al. (2010) describe large changes in deep-water convection patterns over glacial/interglacial cycles as well as the re-routing of currents. The carbonate mounds (overgrown by thickets of cold-water corals) thriving in the Porcupine Seabight on the Irish continental margin occur in the depth range affected by the Mediterranean Outflow Water (MOW, Freiwald, 2003), and the MOW is most likely the important link between climate/sea-level changes and the presence and growth of the carbonate mounds in the region. Dorshel et al. (2005) proposed an evolutionary model for carbonate mound growth in the Porcupine Seabight. **During intermediate periods** (i.e., before the following glacial period), food supply is increased by intensified bottom currents probably due to northward flowing MOW. The density contrast between the MOW and the

Eastern North Atlantic Water (ENAW) may induce the development of internal waves and tides (Pingree and Le Cann, 1990) that again produce locally enhanced bottom currents. This increased water movement that enhances food transport to the corals, baffles / stabilizes the sediments (Frederiksen et al., 1992) and therefore promotes the mounds growth. **Under glacial conditions**, the sea level was ~120 m lower, and therefore the exchange between the Atlantic and the Mediterranean through the Strait of Gibraltar was reduced. Especially, the MOW did not reach further north than 40°N during the Last Glacial Maximum. The MOW and Eastern North Atlantic Water (ENAW) became replaced by a homogenous water mass. Reduced bottom currents, enhanced input of fine sediment and ice-rafted detritus (IRD) resulted in an unfavourable setting for the corals (Freiwald, 2003). Under such conditions, it is assumed that a layer of glacial sediments covered the mound and the corals. **In the course of deglaciation**, the sea level rose and the MOW flowed back in the Porcupine Seabight. The enhanced bottom currents displaced the unstabilized fine-grained glacial sediments and left the coarsest material in place. Then, the continuous presence of strong bottom currents hampered the deposition of fine interglacial material but coral growth was supported.

Actually, recent studies on reef growth in Europe indicate sensitivity to many different **factors such as riverine input, nutrient levels, salinity, temperature** (Freiwald, 2003; Duineveld et al., 2004; Roberts et al., 2006) and the **effect of lower sea levels, which are all modified during the glacial/interglacial periods**. As shown by Dullo et al. (2008), the combined influence of both **temperature and salinity** is important, with living cold-water corals from the Irish and the Norwegian margin closely following a potential density isopycnal surface of sigma–theta=27.5 +– 0.15. Reef growth and the factors responsible for it have been well studied in the **Mediterranean Sea**. There, the main phase of deep-sea coral growth occurred within the Younger Dryas (YD) (i.e. from 12,850 to 11,820 years BP) period, that marks (i) a brief ~1200 years interruption to the warming of the last deglaciation (~19,000 to 6000 years BP) and therefore (ii) a return to cooler ~12 °C to 13 °C Sea Surface Temperatures similar to those in the Late Glacial Maximum (i.e., cold reversal), as well as (iii) nutrient-rich bathyal regimes (McCulloch et al., 2010). The most likely **source of increased nutrients** into the Mediterranean during the mid and post-YD periods is inferred to be **from increased river input from glacial melting** (Barcena et al., 2001). It is then suggested that the demise of coral mounds in the West Mediterranean Sea was possibly due to the rapid burial by sediment-rich meltwater flow into the Mediterranean that accompanied the

final glacial retreat in northern Europe, causing the destruction of the benthic habitat. Similar burial events (i.e., ice-rafting events) have been documented during the Holocene in the North Atlantic (Bond et al., 1997).

However, one might wonder if allopatric speciation is the only reason why coral reef environments (shallow or deep) host some of the most diverse species assemblages on the planet. The alternatives, speciation with **complete (sympatric)** and **partial (parapatric)** range overlap might also explain some of the high biodiversity reported in such ecosystems. First proposed by Darwin (1859), natural selection plays a role in the development of reproductive isolation through the ecological interactions of organisms with their environment (e.g., ecological speciation). Studies from marine systems show that natural selection caused by sharp environmental discontinuities can cause really rapid divergence and may eventually result in speciation (see Palumbi, 1994). It is hence likely that non-allopatric speciation plays a role in the generation and maintenance of biodiversity in deep-sea coral ecosystems. Actually, recent studies in shallow-water coral ecosystems (e.g., corals, sponges, molluscs and fishes) show that **natural selection can strongly influence diversification processes**, and ultimately may be as important as geography in driving speciation (Rocha et al., 2007). Rocha (2005) showed that selection by habitat in shallow-water coral reefs might be an important source of diversification in fishes. The marine sponge *Chondrilla* cf. *nucula* exhibits important genetic divergence between adjacent mangrove and reef populations, but significant similarity among broadly separated populations within the same habitat (Duran and Rützler, 2006). Then, Carlon and Budd (2002) revealed that important genetic differences between two morphotypes of the coral species *Favia fragum* co-occurring in the same reefs in Panama, were apparently maintained by strong natural selection; one of the types was shown to prefer shallow waters with strong fluctuations in salinity and turbidity, while the other inhabits a deeper and more stable environment. These examples illustrate that habitat preference might be more important than dispersal ability in coral reefs, and that we must therefore also consider causes such as physical, environmental or selective mechanisms that differentiate reef associated populations.

Although some cryptic species of *Hexadella* and *Plocamionida* were found allopatric, sibling taxa were mostly reported to occur in sympatry. Cryptic taxa were separated by low

(i.e., 0.5% between the *Plocamionida* lineages H7/H8 in the Rockall Bank) to high genetic divergence (i.e., 4.2 % between the *Hexadella* lineages H3/H1 in Ireland or 15.5% between the *Plocamionida* ESUs H3/H8 in the Gulf of Cadiz), which may represent young to old events of speciation. The implications of these findings are of great importance, as one might wonder if these **sympatric sponge species occupy different undetected niches in cold-water coral ecosystems**? It is indeed often assumed that very recent species compete for the same resources, while very divergent ones show resource partitioning (e.g., Pianka, 1969). Although sponges are traditionally viewed as unselective filter feeders, Maldonado et al. (2010) showed a selective feeding of the marine sponge *Hymeniacidon perlevis* on pathogenic microbes, at different rates and following different cellular mechanisms. As discussed in CHAPTER I, many hypotheses aiming to explain the high species diversity in bathyal environments, refer to time-stability and seasonality. First, small-scale patches of food, biogenic structures, and disturbance create a habitat mosaic (small-scale niche heterogeneity) which potentially causes discordant sponge distribution (i.e., species distributed in separated patches) and allows the coexistence of species. Then, such wide range of habitat, from large to small scales (some kilometers to meters or cm) described in the deep sea may promote shifts in ecological parameters or invasions of new habitats which may cause the processes of local adaptation and natural selection to act at really small scales. As such, local and regional scale processes may indeed be ultimately reflected in phylogenetic diversification over an evolutionary time scale. Here, the co-existence of closely related sponge species in the deep-sea coral ecosystems constitutes further evidence that species might show responses to/ benefit from small environmental differences in bathyal habitats. In addition, random and moderate environmental fluctuations, in contrast to the high energetic shallow-water environments, avoid attainment of equilibrium and competitive exclusion. As such, even if species explore the same resources, random disturbance may reorganize the local species structure before competitive exclusion leads to extinction and reduces diversity (Tilman and Pacala, 1993).

Cold-water coral reef patches, with their mosaic of dead, live, and decaying corals and associated species, embracing each other at really small scales, are here suggested to be important sources of biotic structure and habitat heterogeneity in the deep sea. Under-water seabed photographs and ROV videos of dense cover of living cold-water corals, attached to a

thick layer of coral debris, give a true representation of this physical heterogeneity. It is also suggested that dead coral blocks contain several different habitats (Jensen and Frederiksen, 1992). The question that remains is how small such microhabitats could be? We hypothesize that cold-water corals constitute unique environments, holding a potentially wide range of multiple microhabitat (i.e., cm^2 in area) for sponges. In addition, virtually nothing is known about small-scale vertical distribution of niches in the deep sea**.** Depth was shown to structure sponge communities (Longo et al., 2005; Van Soest et al., 2007a) within the same cold-water coral ecosystem. We can therefore question whether such a parameter may play a role in the high species diversity of Porifera in the deep-sea coral reefs. Also, a question that remains is to which extent do sponge species interact with live corals and what is the nature of such interaction (e.g., competition, mutualism, trophic interaction)?

In any case, the biogeography of sponges probably reflects some combination of both environmental heterogeneity and dispersal limitation (i.e., history). These two hypotheses should probably be seen as endpoints of a continuum, where the reality for many taxa might lie in between.

The molecular markers and their limit in sponge diversity studies

COI

The mitochondrial cytochrome c oxidase subunit 1 (COI) was a marker of choice for the 'Barcoding of Life' initiative (i.e., the proposition of using the first 650 bp of the COI gene to identify all living species, Hebert et al., 2003). In *Hexadella*, minimum genetic divergence values between the three supported COI clades (H1–3) and the two divergent sequences (Seq1,2) ranged from 3.9 % to 8.7 % (corrected p-distance) whereas in *Plocamionida,* genetic divergence values among ESU's varied from 1.3 % to 20 %. Although the low COI genetic differences between some of the *Plocamionida* ESU's (CHAPTER V: 1.3 to 1.9 %) were clearly smaller than the interspecific distances found within other genera, such as *Scopalina* (Order Halichondrida, 11 to 22 %; Blanquer et al., 2007), Poppe et al. (2010) reported very low genetic distance values (maximum 1.8 %) between morphologically distinct *Psammocinia* species (Order Dictyoceratida).

Consequently, the **COI marker was confirmed to show different levels of genetic variation between different sponge taxa** and these results emphasize the problem of defining a threshold to delineate species in the phylum Porifera. As for other organisms, **sufficient data on intra- and interspecific variation of the COI gene** (and of any other marker), is therefore required to delineate species (See Vogler and Monaghan, 2006).

Erpenbeck and colleagues (2006b) showed that the COI downstream I3M11 partition provides more resolution than the standard barcoding fragment, called the M1M6 partition. The I3M11 partition proved useful at inter-specific level (Xavier et al., 2010a) and to determine the genetic population structure of Caribbean and European sponge species (Lopez-Legentil and Pawlik 2009; Xavier et al., 2010b). Here, we attempted to compare the COI I3M11 partition to the M1M6 partition in *Plocamionida* species in terms of amplification success and nucleotide variability/substitution pattern, as well as to assess the potential of their combination for species level delineation. A slightly lower ratio of transitions/transversions (1.45 vs. 1.941) was found in I3M11 vs. M1M6 partitions for our *Plocamionida* specimens (27 samples). Moreover, the I3M11 partition was much easier to amplify than the M1M6 partition, because sequences of the M1M6 partition were often impeded by contaminations (hydrozoans, microbial symbionts, etc.). Our study also confirmed the resolution power and suitability of the I3M11 COI partition for low level phylogenies such as barcoding (Sponge Barcoding project, http://www.spongebarcoding.org/), as the same number of *Plocamionida* ESU (eight) was detected using the M1M6 and I3M11 partitions separately or jointly. The higher amplification success and variability of I3M11 adds to the growing evidence that it may be a better COI partition than M1M6 to infer interspecific diversity, at least for those groups for which M1M6 has proven to be difficult to amplify (for Nematoda see Derycke et al. 2010, for sponges see this study). At the intra-specific level within the deep-sea species *Plocamionida microcionides*, the I3M11 COI partition showed a relatively high genetic variability between three northern populations with a maximum uncorrected p-distance of 1.7 % across a distance of 800 to 2000 km. In addition, the overall nucleotide diversity (π = 0.003988) was an order of magnitude higher than for previous studies at similar or larger spatial scales using the M1M6 COI partition (e.g., π = 0.00049, Wörheide 2006; π = 0.0006, Duran et al., 2004; π = 0.00058, Lopez-Legentil and Pawlik 2009). In contrast, nucleotide diversity was of the same magnitude as that found in recent intraspecific sponge studies using I3M11 (π = 0.0039,

Lopez-Legentil and Pawlik 2009; π = 0.0042, Xavier et al., 2010b). It shows an informative genetic variation of the COI I3M11 partition and highlights the utility of this alternative COI partition for intraspecific analyses within *P. microcionides*.

For the *Plocamionida* phylogenetic analyses in this study, a ~1200 bp long fragment of the cytochrome c oxidase subunit I (COI) mtDNA gene was initially amplified from ten random specimens using the universal primer LCO1490 (Folmer et al., 1994) and the reverse primer COX1-R1 (Rot et al., 2006). Based on these ten sequences, specific *Plocamionida* sp. primers COIPlo20F and COI800Rev were developed to amplify the M1M6 partition. PCR amplifications of the I3M11 partition were performed using the primers COI800Fwd (reverse complement from the specific primer COI800Rev) and COX1-R1. These studies show that although the use of 'universal' primers such as those of Folmer et al. (1994) is useful for the amplification of some samples, the use of taxon-specific primers has obvious methodological advantages. Indeed sponges are a group that are known to host diverse microbial communities (Taylor et al., 2007) and amplification with universal primers ended in many contamination products like proteobacteria but also other macro organisms such as hydrozoans. The primers we developed, although they amplify a restricted range of sponge species belonging to the same order, gave high confidence rate. We therefore highly recommend the development of such taxon-specific primers for low level phylogenetic studies in this taxonomic group. Because of the enhanced chance of amplifying symbionts or ingested DNA templates in encrusting sponges, COI sequences should also be systematically verified for their poriferan origin by BLAST searches against the GenBank database (http://blast.ncbi.nlm.nih.gov/ BLAST/) and with a cladistic tree-reconstruction as described in Erpenbeck et al. (2002).

28S

One of the first molecular phylogenies on sponges used the 28S rDNA gene (partition C1-D2, Lafay et al., 1992). Although C1-D2 proved informative to resolve poriferan intra-ordinal relationships (Borchiellini et al., 2004a), D1 was not adequate to resolve some inter-family relationships (e.g., Astrophorida, Borchiellini et al., 2004b). Other studies have been using other partitions, such as D3-D5 (e.g., Mc Cormack and Kelly, 2002; Erpenbeck et al., 2004), which proved useful for high-level phylogenies. Here, we used the D3-D5 partition of the 28S rDNA for inter-specific studies. In the genus *Hexadella*, the phylogenetic resolution in the nuclear ribosomal 28S gene was about 10-fold smaller than for COI and 25-fold smaller

compared to ATPS. The 28S marker proved useful to corroborate the genus *Hexadella* as a well-supported monophyletic group, although it was unable to discriminate the different *Hexadella* species. On the other hand in *Plocamionida*, phylogenetic relationships using the 28S fragment were highly similar to the ones obtained from COI. The 28S, in combination with COI, was found suitable to highlight putative cryptic species within *Plocamionida* (e.g., *H1-3* and *H7-8*). These results, as for COI, confirmed the different resolution power of the nuclear 28S marker among divergent taxa and the taxa-specific rate of evolution of this gene. Erpenbeck et al. (2004) already showed the existence of significant differences in 28SrRNA secondary structure and evolutionary rates among taxa, which might bias phylogenetic signal and phylogenetic reconstructions. rRNA molecules indeed undergo a complex folding into a secondary structure of single-strands ('loops') and partially doublestrands ('stems' or 'helices'). This pattern provides the final ribosome a crucial shape for its functionality. These different parts of the 28S molecule do not undergo the same functional constraints throughout the molecule, nor throughout the demosponge taxa (Erpenbeck et al., 2007). Here, the suggested higher evolutionary rates in 28S for the species *Plocamionida* (0.1% to 3.3% of genetic divergence) compared with *Hexadella* (max. 1.8% genetic distance), in addition to the increased evolutionary rates in mitochondrial DNA, could perhaps allow members of this taxon to open up more ecological niches, and cause a higher species biodiversity in the genus *Plocamionida* than in *Hexadella*. Furthermore, although we used a single model of substitution in our ML and Bayesian reconstruction methods for the 28S gene, Erpenbeck et al. (2007) highlighted that traditional approaches, which do not consider the character dependence within 28S, are outperformed by secondary structure-specific substitution models. In any case, these issues suggest additional gene trees based on other, independent genes to be necessary for the investigations of phylogenetic relationships in the phylum Porifera.

ATPS

The second intron of the nuclear ATP-synthetase beta subunit gene has recently been shown to provide a high resolution at the intraspecific level in sponge evolutionary studies (Bentlage and Wörheide, 2007; Wörheide et al., 2008). The ATPS marker was tested here for the first time for its applicability in sponge species delineation; we used the second intron of the nuclear ATP-synthetase beta subunit gene (ATPS) to delineate the European *Hexadella* species, and to reveal the presence of cryptic species within the genus.

On the one hand, the minimum ATPS genetic divergences between the clades/divergent sequences were about twofold the values found with COI, and ranged from 10 % to 28.5 %. Our analysis proved that the ATPS intron marker is useful for taxonomic purposes within the genus *Hexadella* and suggested that its use may be of great interest as a complement for mitochondrial markers in sponge molecular studies. In this study, where the nuclear gene ATPS was used for taxonomic and phylogenetic purposes, it was not required to resolve the putative two different allele copies within individuals, such as for analyses at the population-level. Chromatograms of both forward and reverse sequences were checked for length and sequence variants (to ensure unambiguously alignable sequences). No length variation was observed within individuals and few ambiguous positions (i.e., at most three positions out of 235 bp, representing a maximum ambiguity of 1.3%) were easily encoded using the IUPAC ambiguity code (The International Union of Pure and Applied Chemistry; Cornish- Bowden, 1985).

On the other hand, due to high evolutionary rates, it was difficult to find an adequate outgroup; ATPS intron sequences obtained from closely related Verongida species were unalignable and therefore, midpoint rooting was used in the phylogenetic reconstruction of the ATPS fragment. In addition, the fragment of the nuclear ATPS amplified using the sponge specific primers ATPSb_Ph_Fwd and ATPSb_Ph_Rev (Bentlage and Wörheide, 2007) was small (i.e., 235 bp). Therefore, we had to use the degenerated exon primed intron crossing (EPIC) primers ATPSBF1/ATPSBR1 (Jarman et al., 2002) on some random specimens to obtain the largest possible fragment of the flanking exons of the ATPS gene (Bentlage and Wörheide, 2007) and to facilitate sequence verification by BLAST search (Altschul et al., 1990). These yielded a bigger EPIC-PCR fragment, containing enough bp at both 5' and 3'extremities of the exon sequence. These longer sequences were then checked for their identity with the shorter fragments amplified with the sponge specific primers and their homology with sponge sequences was investigated using a BLAST similarity search (Altschul et al., 1990) with the sequences published by Wörheide et al. (2008). These laborious and double sequencing steps therefore illustrate the difficulty to use the nuclear ATPS markers for phylogenetic studies. Furthermore, two ATPS haplotypes instead of five in the COI dataset were shown in a clade (i.e., H3), possibly because a highly variable position of the ATPS partition showed some double peaks in the chromatogram and could not be resolved. It indicated that the ATPS marker might be used for future phylogeographic studies within

Hexadella species and for deeper insights into gene flow patterns, provided that the two alleles of the intron are retrieved. In addition in other species such as *Desmacella inornata*, *Mycale lingua* or *Poecillastra compressa* (data not shown), we could not amplify the ATPS gene of most individuals and for some positively amplified samples, sequence variation within the genus was too high to allow their alignment.

Unravelling sponge biodiversity through a combination of molecular markers

These studies confirmed that although a DNA barcoding approach (i.e., theoretically a rapid, accurate, automatable species identification by using a short, standardized gene region such as the mitochondrial COI gene) seeks to enhance biodiversity studies, a single marker system is problematic for classical taxonomy of sponges. A gene tree is indeed only a phylogenetic hypothesis, reflecting the specific peculiarities of the gene fragment recruited for phylogenetic reconstructions but not necessarily the species tree. First, our results further exemplify that the COI marker has different rates of evolution in different sponge species. Therefore knowledge on the intra- and inter-specific variation of COI is highly needed. It is now accepted that the genetic patterns observed in some populations may not correspond to the species evolutionary history, but rather to the history of the mitochondria (Ballard and Whitlock, 2004). The interpretation of mtDNA data alone should be done with caution, and nuclear DNA should be analyzed together with mtDNA, especially in cases where hybridization is suspected (e.g., by the presence of highly divergent mtDNA types within species). In the present studies, the same tree topology in independent markers confirmed the consistency of the arrangement and therefore gave confidence in species boundaries delineation as well as in establishing phylogenetic relationships. Phylogenetic analyses in *Hexadella* (using the COI-ATPS-28S dataset) and in *Plocamionida* (using COI and 28S) showed that the combination of at least two independently evolving molecular markers was a useful tool for screening biological diversity, especially in such complex taxa. This generally implies that heterogeneity of rates across genes are handled by separate models in a partitioned analyses (see CHAPTER III) and for more accuracy that rates between different partitions are unlinked.

How an Integrative taxonomy outperforms traditional approaches for the delineation and description of species

The 'Integrative taxonomy' approach, using multiple, complementary data (DNA barcodes, comparative morphology, phylogeography, ecology, development, behavior, etc), is currently recognized as essential for carefully testing species limits (Dayrat, 2005; Will et al., 2005). The study of morphological and biological information indeed represents an extra power to a robust framework investigating species status. In many cases, the species discovered using molecular methods remain in taxonomic crypsis, as there are no known morphological differences in addition to the very strong genetic signal that supports cryptic species. Whether the description of species by molecular sequence (i.e., DNA-based species descriptions) is valid or not has been recurrently debated (See Vogler and Monaghan, 2006). Some authors, although they do not advocate for species descriptions based solely on genetic characters, conclude that there is no compelling reason within nomenclatural rules to forbid such descriptions (Cook et al., 2010). In these cases, DNA-based species descriptions would require tissue and DNA vouchers to be deposited and being available, allowing subsequent analyses.

Here, we provide further evidence that a formal description using morphological data eventually makes new species (i.e., identified through molecular phylogenetic studies) available to the scientific (e.g., ecologically-oriented) community. Recent studies already showed that the status of newly discovered cryptic sponge species can be subsequently validated by investigating additional diagnostic characters, previously neglected or seen as regular intra-specific variation (e.g., Blanquer and Uriz, 2008). Similarly, the *Hexadella* study in CHAPTER IV illustrated how a wide range of characters, including molecular data, morphology, and biochemistry need to be integrated to assess species boundaries and to delimit species in the Phylum Porifera. Our work showed **that only the use of a combination of complementary tools can provide relevant descriptions for some problematic taxa.** This study showed that each taxonomic feature taken apart can not reveal significant differences for all studied species. Although time consuming, the concert of methods applied in our study allowed to point out differential characters between cryptic species. Thus, it exemplifies that the analysis of a great number of specimens in a comparative way enhances taxonomic diagnoses, and therefore implies robust and rigorous classifications.

In addition, although the description of new species can be a laborious and long process for some taxa (i.e., a whole revision of the genus should be performed), an *a priori* or *a posteriori* comparison of morphological data with (congruent) molecular phylogenies can provide the taxonomist insight into potential homoplasies and ‘‘good’’ apomorphic characters (Erpenbeck et al., 2006a). In CHAPTER V, the phylogenetic units were morphologically analyzed to investigate whether concordant molecular lineages are also morphologically distinct and to resolve the current taxonomic difficulties in *Plocamionida*. These analyses showed that the combination of molecular data and morphological characters proved useful for differentiating *Plocamionida* species and for establishing their phylogenetic relationships. Comparing the limited number of morphological characters against a robust and comprehensive phylogenetic (DNA) tree approved to be a fruitful approach for integrating the strengths of morphological data with those of sequence data. The putatively new *Plocamionida* species are now submitted to the filter of other approaches and further sampling followed by detailed phenotypic diagnostic analyses may support the observed molecular differences.

The use of other sources of information such as biochemical (e.g., Loukaci et al., 2004) or ecological data (e.g., Wulff, 2006) was already shown to complement morphology for the delineation of species in the phylum Porifera. Also in case of morphological stasis (i.e. selection forces morphological traits to be conserved), complementary tools (i.e., behavior, chemicals) may be crucial for the detection of species. One explanation for the lack of morphological differences in spite of genetic divergence is that marine organisms communicate through reproductive signals via non visual mating (e.g., sound, vibration, pheromones or electrical signals) and that inter-specific differences in these features rarely leave a morphological imprint (Bickford et al., 2007). Changes in such expressed signals may not need to involve morphological changes, and may result in cryptic species. Our results from CHAPTER IV may support this hypothesis, as the sister taxa *Hexadella racovitzai* and *H. topsenti* sp. nov. were separated based on clearly divergent secondary metabolite patterns within each of the two species. *H. topsenti* individuals were also found to contain significantly less toxins (in contrast to *H. racovitzai* individuals) based on the Microtox bioassays. Actually, general toxicity bioassays integrate the response of all the metabolites present in the organisms, providing a holistic view of the chemical ecology of the species (See Marti et al., 2003).

To conclude, these studies illustrate that cryptic species do not need to have a recent origin because of their similar morphological traits but may have an ancient origin instead. Such ancient divergences were found among many cryptic *Hexadella* and *Plocamionida* species. Although small sized and morphologically cryptic sponges are difficult to identify (Van Soest et al., 2007a), our studies on *Hexadella* add to the growing evidence that genetic differences supported by minute but consistent diagnostic characters (e.g., morphological, biochemical, cytological, toxicity, etc) may allow the description of sponge species.

What are the implications of the presence of cryptic sponge species?

The results of this study confirm that understanding and quantifying biological diversity is imperative if we want to be able to explain and, ultimately, conserve it. First, knowledge on speciation within sponges from shallow and CWC ecosystems has potentially great implications for the efficient conservation and economic management of this group. These studies illustrate the under-estimated diversity of sponges in shallow and especially in bathyal CWC ecosystems along the European margins. The occurrence of cryptic species complexes is important for conservation planning. Harmon et al. (2009) illustrate the effects of evolutionary diversification of fish species on ecosystems, where the so-called 'generalist' species are in fact cryptic complexes of specialists. It is indeed assumed that cryptic species can have a different effect on the ecosystem. Although the function of the cryptic sponge species in shallow-water and cold-water coral ecosystems is still unknown, their role might be different and important for the functioning of ecosystems. Cryptic sponge species might then be essential to consider in bioassays. In terms of bioprospection, the detailed study of species limits in sponges can benefit the search for new pharmaceuticals. Sponges hold natural libraries of novel chemicals with potential medicinally important properties, such as antibiotic or antiviral activity (Munro et al., 1999; Blunt et al., 2011). Because these products vary among species, the occurrence of undetected cryptic species complexes can impede the discovery or exploitation of potentially valuable sources of medicinal compounds. Our data on the species complex *H. racovitzai* (CHAPTER IV) exemplifies these issues, as the newly identified species *H. topsenti* sp. nov. showed a different biochemical profile and significantly different toxicity values.

Avoiding further **habitat loss** might be the greatest challenge for the protection of global biodiversity. Where to place Marine Protected Areas (MPAs) and how they should be spaced is a recurrent question (Halpern et al., 2006). On the one hand, the protection of the largest number of species (including unique lineages) is widely admitted and prioritizing sites for conservation often relies on the estimation of species richness and endemism. It is suggested that the discovery of **geographical and habitat-related patterns** in the distribution of cryptic species might reveal yet-unknown centers of endemism and diversity that justify the consideration for conservation of particular habitats (Bickford et al., 2007). Here, the distribution of sponge species showed a particularly high diversity of sponge lineages in southern reefs (e.g., the Gulf of Cadiz). Although coral ecosystems in southern reefs are known to flourish in glacial times, whereas major periods of coral growth occurred during the Holocene and earlier interglacial periods in higher latitudes, Wienberg and colleagues (2009) interestingly note the abundance of scleractinian corals in the Gulf of Cadiz during the past 48kyr. Therefore, although these southern reefs currently show few live corals, they may hold remnants of unique lineages of deep-water coral ecosystems associates, which survived glacial periods. The region of the Gulf of Cadiz, rich in species diversity might therefore be important to consider for further conservation efforts on cold-water coral reefs.

On the other hand, identifying the **processes that produce biodiversity and influence marine evolution** is also important. Estimating dispersal is needed to promote both effective species protection and spill-over to non-protected areas (Palumbi, 2004). However, there is a great gap in knowledge for deep-sea marine organisms. We suggest in this study that both geography and natural selection may shape the genetic architecture of species in cold-water coral reefs. Protecting 'ecological hotspots' (Rocha et al., 2007) might imply to consider MPA's in **geographically isolated coral reefs as well as among coral reefs with divergent ecological conditions** (i.e., areas which are in close geographic proximity).

Future directions

Deep-sea diversity estimation

The deep-sea fauna is indisputably diverse, but the question of how diverse remains unanswered. Grassle and Maciolek's (1992) benthic extrapolation of at least 1 million and

potentially more than 10 million species is apparently at the upper end (Ramirez-Llodra et al., 2010). In order to provide a better species richness estimate in the deep-sea, cases of extremely well-sampled and identified taxa, in both deep-sea and shallow-water systems may be used to better extrapolate species richness. The challenges that remain are hence i) to increase the sampling scheme in shallow waters and the deep sea for these sponge phyla; ii) to identify potentially new species at a regional or global level, and to provide species description.

Comparative phylogeographic studies among CWC communities from eastern to western Atlantic

A better understanding of connectivity among CWC communities and associates will depend on studies that encompass all spatial scales of geographic isolation, and thus will require collaborative research efforts. Conducting studies on several different members of the coral community will indeed allow for identification of 'common' or 'overlapping' phylogeographic breaks in the North Atlantic, which is critical information to design MPA networks. It is believed that when climate warmed after the end of the Last Glacial Maximum, species from southern refugia moved northward, recolonizing Northern Europe, and this fundamental expansion-contraction (EC) model provides a simple representation for the demography of species through glacial cycles. It however tends to be more complicated due to issues such as different species having different environmental niches, distinct dispersal rates and life history traits or interactions between communities of species (Hewitt, 1999). As with many phylogeographic approaches which aim at reconstructing the past history of organisms, the concerted information provided by comparative studies can give true insights into the larger-scale forces that shape the current-day distributions of species (Provan and Bennett, 2008). Data from comparative phylogeographic studies, including other sponge species but also other phyla, such as the Polychaeta *Eunice norvegica* (commensal of *Lophelia pertusa*), across the same geographic range would allow identifying areas of genetic endemism, which may ultimately help to make decisions about where to focus conservation efforts.

We are planning the additional sampling of *Hexadella dedritifera* (and/or *Plocamionida microcionides*) individuals during (i) the deep-sea campaign Bobeco (Bay of

Biscay and Porcupine Seabight in September-October 2011, Ifremer, ROV Victor) and (ii) the deep-sea Belgica campaign (Bay of Biscay, UGent, June 2011, boxcores targeting key areas for cold-water coral, highlighted in CHAPTER II). We also aim to complete the sponge datasets for phylogeographic analyses and population genetic structure studies in the eastern Atlantic. It is also being proposed that such analyses would be standardized between partners working on the western Atlantic and the Gulf of Mexico, and combined for a more complete assessment of trans-Atlantic gene flow. This project intends a basin-wide/trans-Atlantic comparative study on living CWC systems aiming for a better understanding of the dispersal routes and population dynamics. The genetic structure of CWC species is critical to understand in order to determine the physical scale at which genetic structure is built and therefore to design appropriate conservation plans for coral reef ecosystem biodiversity.

Formal description of the Hexadella cryptic species from the deep sea

The taxonomic revision of Atlanto-Mediterranean *Hexadella* (Porifera, Ianthellidae) using cytology, chemistry and molecular sequences in an integrative approach afforded in this thesis could not include the deep-sea *Hexadella* cryptic complex due to the lack of fresh material for such analyses. Therefore, new individuals of *Hexadella* sp from the above cited campaigns will be transferred immediately into 95 % ethanol for the molecular analysis and in a mixture of glutaraldehyde 2.5 %, 0.4 M cacodylate buffer and filtered sea water (1 vol.: 4 vol.: 5 vol.) for the cytological analysis. These will allow the formal description of taxa from the deep-sea *Hexadella* species complex and to complete the identification key for the Atlanto-Mediterranean *Hexadella* species.

Population genomics of deep-sea sponges associated with CWCs

Full-genome research is presently being conducted for some sponge groups and promises a higher phylogenetic resolution for phylogenetic questions related to the basal evolution of metazoans, including the specific place of Porifera along the Tree of life. In addition, technological advances such as the new generations of sequencers, now promote the emerging field of population genomics to address evolutionary processes at a genomic scale for populations in the wild. These Next Generation Sequencing (NGS) methods are therefore of great promise for the investigation of (i) phylogeographic issues, (ii) local adaptation.

The supposedly neutrally evolving molecular markers (COI, ITS or microsatellites) have typically been used for understanding evolutionary processes in natural populations, via population genetics and phylogeographic analyses. Addressing these processes may however be hampered by issues such as different rates of differentiation across regions of the genome (Hohenlohe et al., 2010) or the action of selection (e.g., Peek et al., 2000). Instead of analyzing a limited number of genes, NGS allows screening much larger levels of genetic variation (e.g., by using Single Nucleotide Polymorphisms called SNP's which are widely distributed across the genome).

Such wide-genome analyses can also separate gene-specific effects such as selection from whole genome effects (e.g., genetic drift and gene flow). Actually, the ability to detect emerging species may ultimately come from the analysis of genes that are directly under the influence of selection. Signatures of selection can also be used to identify candidate pathways, genes and alleles for targeted functional analyses. These tools might indeed be particularly interesting to study questions related to ecological speciation of sponge species at small to microscale scales in cold-water coral reefs.

Microbial symbiont community analyses

A major research theme that follows the phylogenetic analyses of shallow and deep-sea encrusting sponge species is the comparative analysis of the sponge-associated microbial communities within and between the divergent sponge lineages. The structure of microbial communities in relation to sponge taxonomy (including the numerous cryptic species highlighted), biogeography and ecology is key information for a better understanding of host-symbiont co-evolutionary processes, and may have important implications for the sound conservation of marine resources. Sponges host a large community of micro-organisms including Bacteria, Archaea and Fungi (Taylor et al., 2007). In some cases as much as 40 % of the mass of the sponge may consist of microorganisms. Major recent interest in the sponge-associated microbial community has emerged due to the potential production of a diversity of biologically-active secondary metabolites by these symbiotic associations, which are valuable resources in the biotechnology and pharmaceutical industry. These are also pending questions for the role of sponge-microbial association in ecosystem functioning, as recent studies indicate possible roles of microbial symbionts in sponge chemical defense, health and in situ nutrient cycling (Hentschel et al., 2006). The sponge-associated microbiome (i.e., the

combination of microbial genomes present in sponges), therefore constitutes a valuable reservoir of metabolic and genetic diversity that needs to be investigated. Especially, deep-water sponge species constitute a barely explored reservoir of diversity. Despite the fact that sponges dominate cold-water coral ecosystems, very little is known about the microbial communities associated with the phylum Porifera in these remote environments.

Species of the genus *Hexadella*, which are biochemically and ecologically important, and the phylogenetic relationships of which have recently been unlocked (see CHAPTER III and IV), represent perfect model taxa for such investigations. The widespread occurrence of *Hexadella* species from shallow to bathyal cold-water coral environments along the Mediterranean to Norwegian margins implies a wide range of environmental parameters. Such evolutionary and biogeographical context would allow to study in detail the structure of microbial communities in relation to sponge taxonomy and/or environmental parameters. The microbial community structure (archaeal and bacterial) can be evaluated using amplicon pyrosequencing of 16s rDNA gene libraries (Webster et al., 2010). This high throughput parallel sequencing strategy allows circumventing limitations of less resolving approaches (e.g., DGGE, clone libraries). In addition, functional diversity of microbes inhabiting these sponge habitats could be evaluated when possible, based on taxonomic assignment of the 16s rDNA sequences and comparison with the large 16s rDNA database.

ADDENDA

Addendum **I**

Appendix to CHAPTER II:

Reveillaud J, Freiwald A, Van Rooij D, Foubert A, Le Guilloux E, Altuna A, Vanreusel A, Olu-Le-Roy K, Henriet JP (2008) The distribution of scleractinian corals in the Bay of Biscay, NE Atlantic. Facies 54, 317-331.

Electronic Supplementary Material

Table with station coordinates and sampling depths of scleractinian corals from various research cruises dedicated to the Bay of Biscay, grouped by species.

ADDENDUM I

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Anomocora fecunda				
Zibrowius 1980 (R/V Travailleur 1882)	N.A (= not available)	-5,571	43,983	1000
Balanophyllia cellulosa				
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-3,607	43,615	137-400
Zibrowius 1980 (R/V Jean Charcot 1968)	N.A	-7,547	47,938	214-235
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-9,800	48,450	330
Zibrowius et al. 1975 (R/V La Perle Biogas 1)	3/DS.2	-7,669	47,942	390
Zibrowius 1980 (R/V Thalassa 1970-1971)	X.305	-5,010	44,083	463
Balanophyllia thalassae				
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-3,592	43,613	380
Zibrowius 1980 (R/V Travailleur 1882)	N.A	-5,571	44,067	512
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.304	-5,075	44,083	515-523
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.316	-4,997	44,035	520
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.318	-4,837	44,120	525
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.313	-4,920	44,043	530
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.363	-4,887	44,100	550
Zibrowius et al. 1975 (R/V Thalassa 1972)	Y.434	-8,680	44,200	620
Zibrowius et al. 1975 (R/V Thalassa 1970)	W.392	-4,822	44,115	675
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.342	-4,603	44,125	700
Zibrowius et al. 1975 (R/V Thalassa 1970)	W.404	-5,737	43,955	700-750
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-8,683	44,207	860-1150
Caryophyllia abyssorum				
Monteiro Marques and Andrade 1981	59	-8,705	44,232	600-922
Alvarez-Claudio 1994	Station H1	-5,750	43,917	702
Alvarez-Claudio 1994	Station H5	-5,733	43,968	769
Alvarez-Claudio 1994	Station H4	-6,170	43,775	790
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,493	44,117	860-910
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Zibrowius 1980 (H.M.S. Porcupine 1870)	N.A	-9,300	48,100	986
Zibrowius 1980 (from Duncan 1877)	N.A	-9,067	44,100	995-1006
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,602	48,667	1170
Alvarez-Claudio 1994	Station I6	-5,900	43,953	1186
Alvarez-Claudio 1994	Station I2	-5,900	43,988	1189
Zibrowius 1980 (R/V Job ha Zélian 1967)	N.A	-4,583	46,283	1300-1340
Caryophyllia ambrosia				
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,533	44,175	1520-1910
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-5,203	44,183	1700-1900
Zibrowius 1980	N.A	-8,183	47,617	1830-2940
Zibrowius 1980 (R/V Jean Charcot Noratlante 1969)	N.A	-4,155	44,122	1884-1911
Zibrowius 1985	BIOGAS station 1	-4,250	44,077	1894-2430
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Zibrowius 1980 (from Gravier 1920)	N.A	-9,683	43,758	2320
Zibrowius 1980 (R/V Jean Charcot Gestlante 2 1967)	N.A	-4,100	45,767	2480
Caryophyllia atlantica				
Zibrowius 1980 (R/V Travailleur 1880)	N.A	-4,079	43,592	1107
Zibrowius 1980 (R/V Sarsia 1961)	N.A	-10,283	48,467	1130-1160
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-10,317	48,467	1200-1300
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-9,833	48,467	1300

ADDENDUM I

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-5,217	46,633	1336-1465
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,883	48,692	1420-1470
Caryophyllia calveri				
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-3,137	43,637	200-400
Zibrowius 1980 (R/V Job ha Zélian 1968)	N.A	-3,175	4,250	400-600
Zibrowius 1980 (R/V Thalassa 1968)	N.A	-9,555	43,380	480-590
Zibrowius 1980 (R/V Sarsia 1958)	N.A	-6,500	47,417	586-897
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-9,887	48,662	750
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-8,850	44,143	970-1045
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-7,078	47,550	1050
Caryophyllia cyathus				
Zibrowius 1980 (R/V *Thalassa* 1968)	N.A	-8,702	44,202	695-760
Caryophyllia sarsiae				
Zibrowius et al. 1975 (R/V Jean Charcot 1968)	26	-6,503	47,442	500-600
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.393	-7,078	47,633	750
Zibrowius et al. 1975 (R/V Sarsia 1971)	1	-6,600	47,317	880-990
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,833	44,025	900-1100
Altuna 1995	Cap Breton	-2,118	43,707	948
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.403	-7,480	47,648	1000
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,630	43,713	1100
Caryophyllia seguenzae				
Altuna 1995	Cap Breton	-2,350	43,724	910
Altuna 1995	Cap Breton	-2,347	43,743	946
Altuna 1995	Cap Breton	-2,118	43,707	948
Zibrowius 1980 (from Roule 1896)	N.A	-2,296	44,283	950
Altuna 1995	Cap Breton	-2,336	43,720	954
Zibrowius 1980 (from Roule 1896)	N.A	-2,413	44,083	960
Altuna 1995	Cap Breton	-2,262	43,731	973
Altuna 1995	Cap Breton	-2,348	43,734	974
Altuna 1995	Cap Breton	-2,284	43,718	978
Altuna 1995	Cap Breton	-2,044	43,596	989
Altuna 1995	Cap Breton	-2,286	43,715	989
Altuna 1995	Cap Breton	-2,343	43,725	993
Zibrowius 1980 (R/V Job ha Zélian 1967-1972)	N.A	-2,767	43,933	1020-1300
Zibrowius 1980 (R/V Job ha Zélian 1967-1972)	N.A	-2,775	44,100	1020-1300
Zibrowius 1980 (R/V Walther Herwig 1975)	N.A	-2,317	44,300	1080-1150
Zibrowius 1980 (R/V Sarsia 1972)	N.A	-3,630	43,713	1100
Zibrowius 1980 (R/V Travailleur 1880)	N.A	-4,079	43,592	1107
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-8,155	47,845	1180
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-10,317	48,467	1200-1300
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-8,143	47,850	1240-1310
Zibrowius 1980 (R/V Travailleur 1880)	N.A	-4,140	43,640	1353
Zibrowius 1980 (R/V Jean Charcot 1967)	N.A	-3,883	45,822	1400
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,533	43,650	1900
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,755	44,123	1930-2100
Caryophyllia smithii				
Zibrowius 1980 (R/V Job ha Zélian)	N.A	-2,022	44,117	118-210
Zibrowius 1980 (R/V Job ha Zélian)	N.A	-4,233	46,500	118-210
Alvarez-Claudio 1994	Station C3	-5,809	43,759	146
Alvarez-Claudio 1994	Station C6	-5,900	43,806	146
Alvarez-Claudio 1994	Station C2	-5,749	43,747	150
Alvarez-Claudio 1994	Station C5	-5,812	43,831	150

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Alvarez-Claudio 1994	Station C7	-5,987	43,806	154
Alvarez-Claudio 1994	Station D2	-5,737	43,802	161
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-1,930	43,650	165
Alvarez-Claudio 1994	Station D3	-5,678	43,854	172
Alvarez-Claudio 1994	Station E1	-6,062	43,878	183
Alvarez-Claudio 1994	Station E3	-5,739	43,710	183
Zibrowius et al. 1975 (R/V Jean Charcot CNEXO 1969)	3	-7,813	48,227	183-184
Alvarez-Claudio 1994	Station F3	-6,079	43,866	227
Zibrowius et al. 1975 (R/V Thalassa 1967)	T.459	-7,810	47,993	235
Zibrowius et al. 1975 (R/V Sarsia 1974)	A3	-9,850	48,717	245-260
Zibrowius et al. 1975 (R/V Sarsia 1957)	37993	-9,700	48,717	247-275
Zibrowius et al. 1975 (R/V Thalassa 1967)	T.438	-7,770	47,977	255
Zibrowius et al. 1975 (R/V Sarsia 1960)	35/2	-10,917	48,917	260-310
Zibrowius et al. 1975 (R/V Sarsia 1974)	A4	-9,867	48,700	275-330
Zibrowius et al. 1975 (R/V Sarsia 1974)	A5	-9,867	48,700	275-340
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-9,600	42,932	280
Zibrowius et al. 1975 (R/V Thalassa 1967)	T.442	-7,855	47,920	290
Alvarez-Claudio 1994	Station G2	-5,879	43,856	300
Alvarez-Claudio 1994	Station F2	-5,972	43,883	307
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.427	-9,807	48,450	330
Zibrowius et al. 1975 (R/V Jean Charcot Biacores 1971)	258	-7,850	47,958	370-347
Alvarez-Claudio 1994	Station G1	-5,656	43,933	468
Deltocyathus conicus				
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,630	43,713	1100
Zibrowius 1980 (Prince de Monaco)	N.A	-9,038	43,892	1674
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Zibrowius 1980 (R/V Sarsia 1972)	N.A	-3,533	43,650	1900
Deltocyathus moseleyi				
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-4,472	44,075	532
Alvarez-Claudio 1994	Station G6	-5,666	43,978	549
Alvarez-Claudio 1994	Station H1	-5,750	43,917	702
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-7,302	47,580	825
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-9,583	42,700	990
Alvarez-Claudio 1994	Station I2	-5,900	43,988	1189
Zibrowius 1980 (R/V Jean Charcot Gestlante2 1967)	N.A	-3,850	45,767	1200
Zibrowius 1980 (R/V Sarsia 1956)	N.A	-7,467	47,633	1300-1372
Dendrophyllia cornigera				
Alvarez-Claudio 1994	Station A1	-5,820	43,682	50
Le Danois 1948	Penmarc'h pointe	-4,383	47,717	90
Alvarez-Claudio 1994	Station B5	-5,983	43,733	121
Alvarez-Claudio 1994	Station C4	-5,908	43,756	130
Alvarez-Claudio 1994	Station C5	-5,812	43,831	150
Joubin 1922	39	-10,867	48,967	185
Le Danois 1948	Cap-Breton	-1,717	43,600	200
Zibrowius et al. 1975 (R/V Thalassa 1970)	W.368	-3,615	43,615	200-400
Zibrowius 1980	N.A	-10,917	48,917	200-500
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-2,098	43,515	200-620
Joubin 1922	87	-4,333	46,367	243
Zibrowius et al. 1975 (R/V Jean Charcot)	14	-7,547	47,938	214-235
Joubin 1922	88	-4,250	46,333	290
Freiwald and Henrich 1997 (R/V Victor Hensen 1997)	VH-97-320	-5,476	46,972	290

ADDENDUM I

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Joubin 1922	55	-5,500	47,050	300
Le Danois 1948	Massif de la Grande Vasière	-4,917	46,833	300
Le Danois 1948	shelf edge	-4,333	46,333	300
Le Danois 1948	Cap-Breton	-2,000	43,667	300
Joubin 1922	75	-4,500	46,583	307
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.427	-9,807	48,450	330
Zibrowius et al. 1975 (R/V Sarsia 1954)	38084	-9,767	48,683	330-366
Zibrowius et al. 1975 (R/V Jean Charcot Biacores 1971)	257	-7,858	47,950	335-355
Zibrowius et al. 1975 (R/V Thalassa 1967)	T.451	-7,845	47,958	358
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.392	-7,022	47,582	390
Zibrowius et al. 1975 (R/V Sarsia 1954)	N.A	-7,317	47,600	394
Le Danois 1948	Massif de la Grande Vasière	-5,500	47,083	400
Le Danois 1948	Massif de la Grande Vasière	-5,333	46,833	400
Le Danois 1948	Massif de la Grande Vasière	-4,667	46,583	400
Le Danois 1948	Le Danois Bank	-5,083	44,000	400
Zibrowius 1980 (from Roule 1896)	N.A	-4,629	46,667	400-500
Zibrowius 1980 (from Gravier 1920)	N.A	-5,392	46,883	446
Le Danois 1948	Le Danois Bank	-5,000	44,033	450
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.402	-7,475	47,658	450
Le Danois 1948	Massif du Banc de la Chapelle	-7,200	47,550	500
Zibrowius et al. 1975 (R/V Thalassa 1972)	Y.429	-8,682	44,197	503
Joubin 1922	62	-4,917	46,833	520
Dendrophyllia alternata				
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-8,683	44,207	450-688
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-9,600	43,105	450-688
Desmophyllum dianthus				
Zibrowius 1980 (R/V Tanche 1921)	N.A	-8,000	47,967	310
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-3,592	43,613	380-1150
Zibrowius 1980 (British Museum 1949)	N.A	-7,083	47,567	440
Freiwald and Henrich 1997 (R/V Victor Hensen 1997)	VH-97-324	-7,326	47,573	530
Zibrowius et al. 1975 (R/V Sarsia 1958)	38000	-6,500	47,417	585-750
Freiwald and Henrich 1997 (R/V Victor Hensen 1997)	VH-97-324	-7,333	47,573	650
Zibrowius 1980 (H.M.S. Porcupine 1870)	N.A	-9,733	48,433	656
Zibrowius et al. 1975 (R/V Thalassa 1968)	U.844	-8,702	44,202	695-760
Monteiro Marques and Andrade 1981	1	-5,097	44,143	676-1350
Alvarez-Claudio 1994	Station H1	-5,750	43,917	702
GALIPOR R/V Belgica 2006	Galor2	-7,207	44,130	720
Alvarez-Claudio 1994	Station H5	-5,733	43,968	769
Zibrowius 1980 (R/V Huxley 1906)	N.A	-7,517	47,600	812
Alvarez-Claudio 1994	Station H2	-5,815	43,755	893
Altuna 1995	Cap Breton	-2,118	43,707	948
Zibrowius 1980 (R/V Travailleur 1881)	N.A	-7,407	44,083	993-1226
Zibrowius 1980 (from Duncan 1877)	N.A	-9,067	44,100	995-1006
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.403	-7,480	47,648	1000
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,630	43,713	1100
Zibrowius 1980 (R/V Travailleur 1880)	N.A	-4,079	43,592	1107
Zibrowius et al. 1975 (R/V Thalassa 1973)	Z.400	-7,317	47,557	1175
Zibrowius 1980 (from Roule 1896)	N.A	-3,546	45,633	1220
Zibrowius 1980 (R/V Job ha Zélian)	N.A	-4,583	46,283	1300-1340
Zibrowius 1980 (Roule 1896)	N.A	-3,913	45,783	1700
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Eguchipsammia cornucopia				

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Zibrowius 1980 (R/V Thalassa 1973)	Z.427	-9,807	48,450	330
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-4,990	44,113	450
Zibrowius et al. 1975 (R/V Thalassa 1971)	X.305	-5,010	44,083	463
Zibrowius 1980 (R/V Sarsia 1962)	N.A	-9,708	48,658	494-567
Zibrowius 1980 (R/V Thalassa 1973)	Z.413	-8,490	48,052	805
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Zibrowius 1980 (R/V Thalassa 1968-1972)	N.A	-9,600	43,105	960
Enallopsammia rostrata				
Zibrowius 1980 (R/V Sarsia 1956-1961)	N.A	-7,467	47,633	915
Zibrowius 1980 (R/V Travailleur 1881)	N.A	-7,407	44,083	993-1226
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Zibrowius 1980 (R/V Thalassa 1970)	N.A	-6,108	43,903	1150
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-7,317	47,557	1175
Alvarez-Claudio 1994	Station I6	-5,900	43,953	1186
Alvarez-Claudio 1994	Station I2	-5,900	43,988	1189
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,883	48,692	1420-1470
Zibrowius 1980 (R/V Sarsia 1956-1961)	N.A	-5,683	47,125	1775
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Flabellum alabastrum				
Monteiro Marques and Andrade 1981	3	-5,025	44,122	800-950
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Monteiro Marques and Andrade 1981	6	-4,752	44,138	900-1000
Zibrowius 1980 (R/V Jean Charcot Gestlante 2 1967)	N.A	-3,883	45,822	1400
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Zibrowius 1980 (R/V Walther Herwig 1975)	N.A	-3,200	44,050	2000-2075
Flabellum angulare				
Zibrowius 1980 (R/V Jean Charcot Noratlante 1969)	N.A	-4,155	44,122	1884-1911
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Zibrowius 1980 (Prince de Monaco)	N.A	-9,683	43,758	2320
Zibrowius 1980 (R/V Travailleur 1880)	N.A	-3,021	43,708	2450-2651
Zibrowius 1980 (R/V Jean Charcot Biaçores 1971)	N.A	-8,783	47,592	2550-2700
Zibrowius 1980 (Prince de Monaco)	N.A	-10,033	43,350	2779
Zibrowius 1985	BIOGAS station 2	-9,017	47,450	3480-3800
Flabellum macandrewi				
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-5,512	43,825	790
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-4,733	44,122	855
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-5,002	44,130	930
Fungiacyathus fragilis				
Altuna 1995	Cap Breton	-1,913	43,607	910
Altuna 1995	Cap Breton	-1,950	43,590	994
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,89	48,61833	1700-1810
Fungiacyathus marenzelleri				
Zibrowius 1980 (R/V Jean Charcot " Noratlante 1969")	N.A	-4,155	44,12167	1884-1911
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Zibrowius 1980 (Prince de Monaco)	N.A	-9,683333	43,75833	2320
Zibrowius 1985	BIOGAS station 2	-9,017	47,450	3480-3800
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,59	44,32333	3860
Zibrowius 1985	BIOGAS station 3	-9,470	47,530	4050-4237
Zibrowius 1985	BIOGAS station 5	-4,822	44,353	4453-4459
Zibrowius 1985	BIOGAS station 4	-10,325	46,455	4550-4825

ADDENDUM I

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Javania cailleti				
Zibrowius 1980 (R/V Sarsia 1958-1974)	N.A	-10,900	48,692	1200-1300
Zibrowius 1980 (R/V Sarsia 1958-1974)	N.A	-5,800	47,200	1200-1300
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430
Zibrowius 1980 (R/V Thalassa 1970)	N.A	-7,770	44,167	2150
Lophelia pertusa				
Joubin 1922	97	-1,717	43,600	150
Joubin 1922	98	-3,767	45,917	150
Joubin 1922	92	-3,767	45,917	155
Joubin 1922	74	-4,333	46,750	162
Joubin 1922	77	-4,333	46,633	162
Joubin 1922	61	-5,133	46,917	163
Joubin 1922	84	-4,750	46,483	163
Joubin 1922	57	-5,333	47,000	170
Joubin 1922	78	-4,333	46,617	170
Joubin 1922	42	-10,583	48,867	180
Joubin 1922	49	-7,301	47,718	180
Joubin 1922	82	-4,583	46,583	180
Le Danois 1948	Massif du Banc de la Chapelle	-7,167	47,500	180-1000
Le Danois 1948	Massif du Banc de la Chapelle	-8,250	48,167	180-1000
Le Danois 1948	Massif de la Grande Vasière	-3,833	45,500	180-1800
Le Danois 1948	Massif de la Grande Vasière	-5,833	47,250	180-1800
Joubin 1922	47	-7,317	47,983	186
Joubin 1922	53	-5,667	47,217	190
Joubin 1922	65	-5,083	46,800	195
Joubin 1922	70	-4,783	46,767	195
Joubin 1922	56	-5,383	47,000	200
Joubin 1922	71	-4,783	46,783	200
Joubin 1922	86	-4,750	46,500	200
Joubin 1922	89	-3,950	46,200	200
Joubin 1922	59	-5,533	46,950	210
Joubin 1922	79	-4,333	46,600	210
Joubin 1922	73	-4,583	46,750	226
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-41B	-7,290	47,599	228
Joubin 1922	44	-8,050	48,167	240
Joubin 1922	40	-10,833	48,967	243
Joubin 1922	41	-10,750	48,983	243
Joubin 1922	54	-5,433	47,167	243
Joubin 1922	58	-5,333	46,967	243
Joubin 1922	51	-7,300	47,600	245
Joubin 1922	48	-7,483	47,783	249
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-41B	-7,290	47,593	251
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-3,600	43,613	260-1150
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-9,600	43,100	260-1150
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-320	-5,476	46,972	285
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-320	-5,473	46,975	295
Joubin 1922	64	-5,233	46,750	300
Joubin 1922	104	-7,117	43,950	300
Le Danois 1948	Massif Cantabrique	-3,833	43,583	300-1200
Le Danois 1948	Massif Cantabrique	-4,167	43,667	300-1200
Le Danois 1948	Massif Galicien de l'Est	-7,000	43,833	300-2000

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Le Danois 1948	Massif Galicien de l'Est	-7,333	44,167	300-2000
Altuna 1995	Cap Breton	-6,710	43,939	308
Zibrowius 1980 (R/V Job ha Zélian)	N.A	-2,100	43,967	320
Joubin 1922	60	-5,150	46,917	324
Joubin 1922	69	-4,917	46,800	324
Joubin 1922	66	-5,000	46,833	330
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-49	-7,246	47,556	340
Joubin 1922	46	-8,000	47,967	350
Joubin 1922	72	-4,667	46,750	354
Joubin 1922	68	-4,917	46,833	356
Zibrowius 1980 (from Jourdan 1895)	N.A	-9,554	43,214	363-510
Joubin 1922	105	-10,083	44,083	366
GALIPOR R/V Belgica 2004	Masma3	-7,287	44,114	381
Joubin 1922	95	-4,750	46,300	388
Zibrowius 1980 (R/V Job ha Zélian)	N.A	-3,117	45,250	400
Joubin 1922	80	-4,167	46,667	400-500
Zibrowius 1980 (from Roule 1896)	N.A	-4,796	46,667	400-500
Zibrowius 1980 (from Roule 1896)	N.A	-4,163	46,667	400-500
Le Danois 1948	Massif Galicien de l'Ouest	-9,000	43,333	400-1800
Le Danois 1948	Massif Galicien de l'Ouest	-9,667	43,833	400-1800
GALIPOR R/V Belgica 2005	Masma5	-7,272	44,112	406
Zibrowius 1980 (British Museum 1949)	N.A	-7,083	47,567	440
Joubin 1922	63	-5,383	46,883	446
Joubin 1922	81	-4,633	46,667	500
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-324	-7,326	47,573	530
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-327	-7,311	47,572	580
Joubin 1922	45	-8,217	48,117	596
Altuna 1995	Cap Breton	-2,236	43,939	635
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-324	-7,333	47,573	650
Joubin 1922	43	-9,733	48,433	654
Joubin 1922	96	-2,117	43,767	677
Alvarez-Claudio 1994	Station H1	-5,750	43,917	702
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-327	-7,317	47,576	720
GALIPOR R/V Belgica 2006	Galor2	-7,207	44,130	720
Alvarez-Claudio 1994	Station H5	-5,733	43,968	769
Joubin 1922	50	-7,517	47,600	811
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Altuna 1995	Cap Breton	-2,118	43,707	948
Zibrowius 1980 (from Duncan 1877)	N.A	-9,067	44,100	995-1006
Joubin 1922	101	-7,158	44,083	1000
Joubin 1922	103	-7,083	44,022	1000
Zibrowius 1980 (from Gourret 1906)	N.A	-5,571	43,983	1000
Joubin 1922	102	-7,208	44,096	1037
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Alvarez-Claudio 1994	Station I2	-5,900	43,988	1189
Zibrowius 1980 (from Gourret 1906)	N.A	-4,038	44,611	1190
Joubin 1922	94	-3,883	45,633	1220
Joubin 1922	100	-7,094	44,083	1226
Joubin 1922	91	-4,017	45,950	1410
Joubin 1922	52	-6,950	47,400	1416
Joubin 1922	90	-4,150	45,983	1480
Joubin 1922	99	-5,883	43,992	1534

ADDENDUM I

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Joubin 1922	93	-3,917	45,783	1700
Joubin 1922	85	-4,633	46,433	1710
Joubin 1922	67	-5,117	46,817	NA
Joubin 1922	76	-4,333	46,667	NA
Joubin 1922	83	-4,700	46,450	NA
Madrepora oculata				
Joubin 1922	97	-1,717	43,600	150
Joubin 1922	98	-3,767	45,917	150
Joubin 1922	92	-3,767	45,917	155
Joubin 1922	74	-4,333	46,750	162
Joubin 1922	77	-4,333	46,633	162
Joubin 1922	61	-5,133	46,917	163
Joubin 1922	84	-4,750	46,483	163
Joubin 1922	57	-5,333	47,000	170
Joubin 1922	78	-4,333	46,617	170
Joubin 1922	42	-10,583	48,867	180
Joubin 1922	49	-7,301	47,718	180
Joubin 1922	82	-4,583	46,583	180
Le Danois 1948	Massif du Banc de la Chapelle	-7,167	47,500	180-1000
Le Danois 1948	Massif du Banc de la Chapelle	-8,250	48,167	180-1000
Le Danois 1948	Massif de la Grande Vasière	-3,833	45,500	180-1800
Le Danois 1948	Massif de la Grande Vasière	-5,833	47,250	180-1800
Joubin 1922	47	-7,317	47,983	186
Joubin 1922	53	-5,667	47,217	190
Joubin 1922	65	-5,083	46,800	195
Joubin 1922	70	-4,783	46,767	195
Joubin 1922	56	-5,383	47,000	200
Joubin 1922	71	-4,783	46,783	200
Joubin 1922	86	-4,750	46,500	200
Joubin 1922	89	-3,950	46,200	200
Joubin 1922	59	-5,533	46,950	210
Joubin 1922	79	-4,333	46,600	210
Joubin 1922	73	-4,583	46,750	226
Joubin 1922	44	-8,050	48,167	240
Joubin 1922	40	-10,833	48,967	243
Joubin 1922	41	-10,750	48,983	243
Joubin 1922	54	-5,433	47,167	243
Joubin 1922	58	-5,333	46,967	243
Joubin 1922	51	-7,300	47,600	245
Joubin 1922	48	-7,483	47,783	249
Joubin 1922	64	-5,233	46,750	300
Joubin 1922	104	-7,117	43,950	300
Zibrowius 1980 (Prince de Monaco)	NA	-7,113	43,950	300
Le Danois 1948	Massif Cantabrique	-3,833	43,583	300-1200
Le Danois 1948	Massif Cantabrique	-4,167	43,667	300-1200
Le Danois 1948	Massif Galicien de l'Est	-7,000	43,833	300-2000
Le Danois 1948	Massif Galicien de l'Est	-7,333	44,167	300-2000
Joubin 1922	60	-5,150	46,917	324
Joubin 1922	69	-4,917	46,800	324
Joubin 1922	66	-5,000	46,833	330
Joubin 1922	46	-8,000	47,967	350

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Joubin 1922	72	-4,667	46,750	354
Joubin 1922	68	-4,917	46,833	356
Joubin 1922	105	-10,083	44,083	366
Joubin 1922	95	-4,750	46,300	388
Joubin 1922	80	-4,167	46,667	400-500
Le Danois 1948	Massif Galicien de l'Ouest	-9,000	43,333	400-1800
Le Danois 1948	Massif Galicien de l'Ouest	-9,667	43,833	400-1800
Joubin 1922	63	-5,383	46,883	446
Joubin 1922	81	-4,633	46,667	500
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-324	-7,326	47,573	530
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-327	-7,311	47,572	580
Joubin 1922	45	-8,217	48,117	596
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-324	-7,333	47,573	650
Joubin 1922	43	-9,733	48,433	654
Zibrowius 1980 (H.M.S. Porcupine 1869)	NA	-9,733	48,433	655
Joubin 1922	96	-2,117	43,767	677
Freiwald and Henrich 1997 (R/V Victor Hensen)	VH-97-327	-7,317	47,576	720
GALIPOR R/V Belgica 2006	Galor2	-7,207	44,130	720
Zibrowius 1980 (H.M.S. Huxley 1906)	NA	-8,217	48,117	755
Alvarez-Claudio 1994	Station H5	-5,733	43,968	769
Joubin 1922	50	-7,517	47,600	811
Zibrowius 1980 (H.M.S. Huxley 1906)	NA	-7,517	47,600	813
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Altuna 1995	Cap Breton	-2,118	43,707	948
Zibrowius 1980 (Duncan 1877)	NA	-9,067	44,100	995-1000
Joubin 1922	101	-7,158	44,083	1000
Joubin 1922	103	-7,083	44,022	1000
Joubin 1922	102	-7,208	44,096	1037
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Joubin 1922	94	-3,883	45,633	1220
Joubin 1922	100	-7,094	44,083	1226
Zibrowius 1980 (Prince de Monaco)	NA	-5,796	47,167	1262
Joubin 1922	91	-4,017	45,950	1410
Joubin 1922	52	-6,950	47,400	1416
Joubin 1922	90	-4,150	45,983	1480
Joubin 1922	99	-5,883	43,992	1534
Joubin 1922	93	-3,917	45,783	1700
Joubin 1922	85	-4,633	46,433	1710
Joubin 1922	67	-5,117	46,817	NA
Joubin 1922	76	-4,333	46,667	NA
Joubin 1922	83	-4,700	46,450	NA
Paracyathus pulchellus				
Alvarez-Claudio 1994	Station B5	-5,983	43,733	121
Premocyathus cornuformis				
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,762	44,118	700-1120
Monteiro Marques and Andrade 1981	3	-5,025	44,122	800-950
Alvarez-Claudio 1994	Station I1	-5,778	44,018	1098
Zibrowius 1980 (R/V Jean Charcot Gestlante 2 1967)	N.A	-3,883	45,822	1400
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Solenosmilia variabilis				
Monteiro Marques and Andrade 1981	1	-5,097	44,143	676-1350

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Zibrowius 1980 (from Duncan 1877)	N.A	-9,067	44,100	995-1006
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,630	43,713	1100
Zibrowius 1980 (R/V Job ha Zélian 1967)	N.A	-4,583	46,283	1300-1340
Zibrowius 1980 (R/V Sarsia 1961)	N.A	-5,217	46,633	1336-1465
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,883	48,692	1420-1470
Zibrowius 1980 (R/V Travailleur 1883)	N.A	-4,146	45,983	1480
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-5,200	44,183	1700-1910
Zibrowius 1985	BIOGAS station 1	-8,417	47,475	1920-2350
Zibrowius 1985	BIOGAS station 6	-4,373	44,188	2430
Stenocyathus vermiformis				
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-8,667	44,200	450-620
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-9,600	43,105	480-540
Alvarez-Claudio 1994	Station G6	-5,666	43,978	549
Alvarez-Claudio 1994	Station H1	-5,750	43,917	702
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-4,472	44,075	750-850
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-9,788	48,637	800
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-8,490	48,052	805
Alvarez-Claudio 1994	Station H2	-5,815	43,775	893
Zibrowius 1980 (R/V Thalassa 1967-1972)	N.A	-9,555	43,380	950-960
Stephanocyathus moseleyanus				
Altuna 1995	Cap Breton Canyon	-1,913	43,607	910
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-5,017	44,130	930
Altuna 1995	Cap Breton Canyon	-2,262	43,731	973
Altuna 1995	Cap Breton Canyon	-2,348	43,734	974
Altuna 1995	Cap Breton Canyon	-2,343	43,725	993
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-7,317	47,557	1175
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-8,125	47,727	1085-1115
Zibrowius et al. 1975 (R/V Thalassa 1973)	N.A	-7,125	47,727	1085-1115
Zibrowius 1980 (R/V Sarsia 1962)	N.A	-10,267	48,492	1200-1300
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-8,100	47,817	1260-1380
Alvarez-Claudio 1994	Station I3	-5,900	43,953	1347
Zibrowius 1980 (R/V Jean Charcot Gestlante 2 1967)	N.A	-3,883	45,822	1400
Zibrowius 1980 (R/V Sarsia 1972-1976)	N.A	-3,533	43,650	1540-1570
Zibrowius 1980 (from Roule 1896)	N.A	-4,663	46,467	1700
Stephanocyathus nobilis				
Zibrowius 1980 (R/V Sarsia 1974)	N.A	-10,317	48,450	1430
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,508	44,168	1520-1600
Zibrowius 1980 (Prince de Monaco)	N.A	-9,096	43,867	1674
Monteiro Marques and Andrade 1981	58	-8,697	44,233	1551-1747
Zibrowius 1980 (R/V Sarsia 1972)	N.A	-3,533	43,650	1900
Zibrowius 1980 (R/V Sarsia 1976)	N.A	-3,767	43,798	1925-1990
Zibrowius 1980 (R/V Thalassa 1971)	N.A	-4,850	44,173	1940
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-10,850	48,578	1975-2070
Zibrowius 1980 (R/V Walther Herwig 1975)	N.A	-3,200	44,050	2000-2075
Zibrowius 1985	BIOGAS station 1	-8,750	47,600	2350
Zibrowius 1985	BIOGAS station 6	-4,373	44,188	2430
Stephanocyathus crassus				
Zibrowius 1980 (R/V Thalassa 1970)	N.A	-8,640	44,175	476
Vaughanella concinna				
Zibrowius 1980 (R/V Thalassa 1973)	N.A	-7,317	47,557	1175
Alvarez-Claudio 1994	Station I6	-5,900	43,953	1186
Zibrowius 1985	BIOGAS station 6	-4,250	44,077	1894-2430

Reference or Cruise	Station Identifier	Long. [W]	Lat. [N]	Depth [m]
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-4,733	44,075	N.A
Zibrowius 1980 (R/V Thalassa 1970-1971)	N.A	-6,108	43,903	N.A
Zibrowius 1980 (R/V Sarsia 1956, 1962, 1974)	N.A	-10,283	48,467	N.A
Zibrowius 1980 (R/V Sarsia 1956, 1962, 1974)	N.A	-7,283	47,633	N.A

Addendum II

(Appendix to CHAPTER V)

Supporting Information

Table S1: *Plocamionida* specimens analysed in the present study. Information regarding the corresponding Evolutionary Significant Unit (ESU), sampling (code, localities with their abbreviation in parenthesis, sampling method, Field Number, voucher specimen, coordinates, depth), number of individuals studied (N) and number of different haplotypes (Nh) for each marker (COI, M1M6, and I3M11 partition, 28S) are provided. Sampling location abbreviations are given in uppercase letters for deep-water samples (>50m) and in lowercase letters for shallow-water samples.

ADDENDUM II

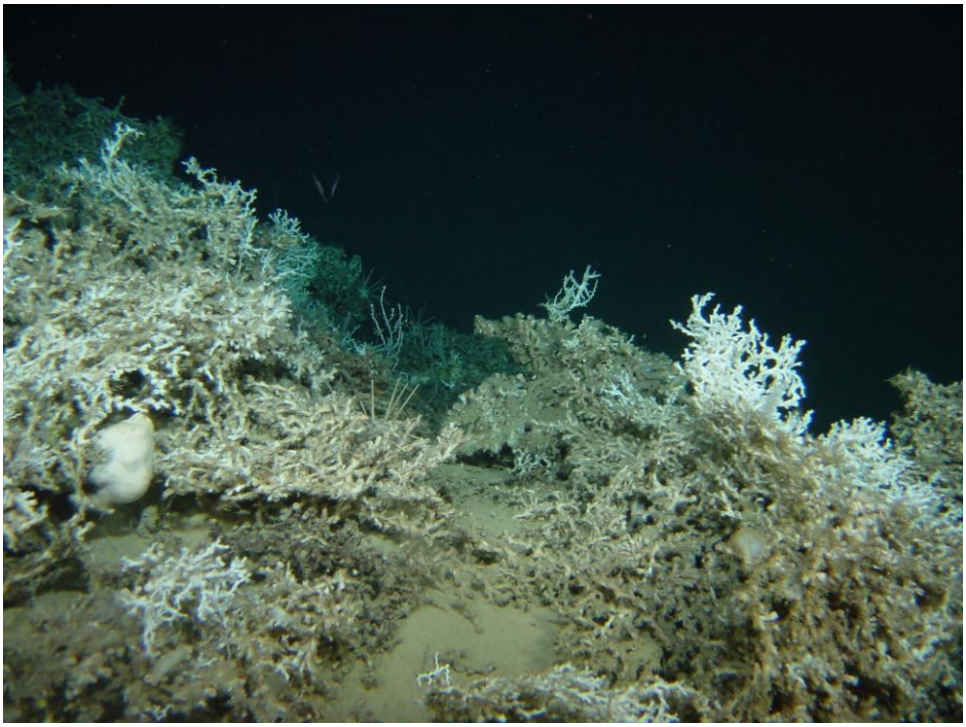
TABLE S1

ESU	SAMPLE	LOCALITIES	Deep-sea cruise, shallow water sampling (collector) or Museum	FIELD NO	Voucher	Lat.(N)	LON.	Depth (m)	NCOI (Folmer)	NhCOI (Folmer)	NCOI (I3M11)	NhCOI (I3M11)	N28S	Nh28S
H8	M2004-02	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/42 Arest	ZMAPOR18566b	55,44	-16,10	777						
H8	M2004-03	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/32E	ZMAPOR18525a	55,50	-15,80	626						
H8	P2	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/32A-05B	ZMAPOR18117	55,50	-15,80	622						
H8	P3	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/33A-14	ZMAPOR.18173	55,50	-15,79	673	2	1	8	4	5	1
H8	P4	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/33A-16	ZMAPOR.18175	55,50	-15,79	673						
H8	P11	Ireland, SW Rockall Bank (ROC)	BIOSYS/HERMES 2005 (R/V Pelagia)	DR40/8	ZMAPOR19527	55,46	-16,02	863						
H8	P14	Ireland, SW Rockall Bank (ROC)	BIOSYS/HERMES 2005 (R/V Pelagia)	BX4/5	ZMAPOR19392	55,50	-15,79	715						
H8	B05-01	Ireland, SW Rockall Bank (ROC)	BIOSYS/HERMES 2005 (R/V Pelagia)	BX156/rest	ZMAPOR20006	55,49	-15,80	573						
H8	P9	Ireland, North Porcupine (POR)	BIOSYS/HERMES 2005 (R/V Pelagia)	DR215/12	no nr.	53,77	-13,95	754	2	2	2	2	2	1
H8	P10 a	Ireland, North Porcupine (POR)	BIOSYS/HERMES 2005 (R/V Pelagia)	DR215/15	no nr.	53,77	-13,95	754						
H8	P23 a	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	VG07/01	no nr.	56,82	-7,40	147						
H8	P25	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	VG35/6	no nr.	56,81	-7,43	149						
H8	P29a	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX123/7	no nr.	56,81	-7,43	162						
H8	P30d	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX127/10	no nr.	56,80	-7,45	82						
H8	P31	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX171/6	no nr.	56,80	-7,45	144	4	2	10	3	9	1
H8	P32b	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX172/3	no nr.	56,80	-7,45	144						
H8	B06-03	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX10/rest	ZMAPOR20259	56,83	-7,40	174						
H8	B06-04	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX48/rest	ZMAPOR20261b	56,81	-7,37	127						
H8	B06-05	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	DR182/rest	ZMAPOR20397a	56,82	-7,37	128-137						
H8	B06-06	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	DR182/rest	ZMAPOR20413a	56,82	-7,37	128-137						
H8	B07-05	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX73/rest	ZMAPOR20475a	59,08	10,74	107						
H8	B07-06	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX73/rest	ZMAPOR20479c	59,08	10,74	107						
H8	B07-07	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX141/rest	ZMAPOR20568c	59,08	10,73	112	1	1	6	1	6	1
H8	B07-08	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX142/rest	ZMAPOR20595a	59,08	10,73	114						
H8	B07-09	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX141/rest	ZMAPOR20563b	59,08	10,73	112						
H8	P22c	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX138/07	no nr.	59,07	10,74	116						
H8	P6	South Portugal, Gulf of Cadiz (CAD)	Moundforce 2004 (R/V Pelagia)	M2004/03-15A	ZMAPOR.18016	35,29	-6,79	531	1	1	1	1	1	1
H7	P12	Ireland, SW Rockall Bank (ROC)	BIOSYS/HERMES 2005 (R/V Pelagia)	BX 48/rest6	ZMAPOR19548	55,50	-15,80	567	1	1	2	2	2	1
H7	P5	Ireland, SW Rockall Bank (ROC)	Moundforce 2004 (R/V Pelagia)	M2004/30-02	ZMAPOR.18488	55,48	-15,87	917						
H6	B07-03	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX73/rest	ZMAPOR20472a	59,08	10,74	107						
H6	B07-04	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX72/rest	ZMAPOR20459b	59,08	10,74	109	3	1	3	1	2	1
H6	B07-10	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX73/rest	ZMAPOR20470	59,08	10,74	107						
H6	MC3539	Ireland, Maidens (ire)	Scuba diving (B.Picton)	no nr.	BELUM:Mc3539	54,93	5,06	42	2	1	2	1	2	1
H6	MC4049	Ireland, Maidens (ire)	Scuba diving (B.Picton)	no nr.	BELUM:Mc4049	54,93	5,07	28,5						
H5	P28	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX119/6	no nr.	56,81	-7,43	129						
H5	P29c	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	BX123/7	no nr.	56,81	-7,43	162	3	1	3	1	3	1
H5	B06-07	Scotland outer Hebrides, Mingulay (MIN)	BIOSYS/HERMES 2006 (R/V Pelagia)	DR182/rest	ZMAPOR20284	56,80	-7,45	144						
H5	MC3992	Ireland, Maidens (ire)	Scuba diving (B.Picton)	no nr.	BELUM:Mc3992	54,94	5,06	33						
H5	MC3982	Ireland, Maidens (ire)	Scuba diving (B.Picton)	no nr.	BELUM:Mc3982	54,95	5,08	34	3	1	3	1	3	1
H5	MC3983	Ireland, Maidens (ire)	Scuba diving (B.Picton)	no nr.	BELUM:Mc3983	54,95	5,05	32,6						
H4	P20b	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX72/08	no nr.	59,08	10,74	109	2	1	2	1	1	1
H4	B07-01	Norway, Skagerrak (SKA)	BIOSYS/HERMES 2007 (R/V Pelagia)	BX73/rest	ZMAPOR20480a	59,08	10,74	107						
H4	BER82-01	Norway, Bergen, Litla Sotra (BER)	ZMA (Zoological Museum of Amsterdam)	no nr.	ZMAPOR4998	60,30	5,10	100	0	0	1	1	0	0
H3	CPOR08-01	South Portugal, Gulf of Cadiz (CAD)	CADIPORIII (R/V Belgica)	CADIPORIII-1	no nr.	35,30	6,14	363	1	1	1	1	1	1
H1	P7	South Portugal, Gulf of Cadiz (CAD)	Moundforce 2004 (R/V Pelagia)	M2004/18-05	ZMAPOR.18055	36,18	-7,32	720	1	1	1	1	1	1
H2	P8	South Portugal, Gulf of Cadiz (CAD)	Moundforce 2004 (R/V Pelagia)	M2004/21-06	ZMAPOR.18068	36,18	-7,31	668	1	1	1	1	1	1

Addendum III

(Appendix to CHAPTER V)

Phylogeography of the deep-sea NE Atlantic *Plocamionida microcionides* (Carter, 1876) (Porifera, Poecilosclerida)



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ADDENDUM III

Introduction

With an island-like distribution, cold-water coral (CWC) reefs raise questions about connectivity and exchange of individuals among geographically separated populations. It indeed remains a critical issue to know if these populations are sustained by larval replenishment or whether they are isolated. A phylogeographic study within the widespread ESU H8 (See Chapter V), corresponding to the species *Plocamionida microcionides* (Carter, 1876) was performed using the COI I3M11 partition to examine the putative connection between populations of CWC reefs associated species. This dataset allowed drawing some tentative phylogeographic conclusions about the connectivity of reef populations along the Northern European margin.

Genetic diversity analysis methodology

Using the COI gene, we performed diversity and genetic structure analyses on populations with more than six specimens of the most widespread species *P. microcionides* (ESU H8). Consequently, the single specimen recovered from the Gulf of Cadiz was not included in our phylogeographic analysis. The intraspecific analyses were performed on the three Northern populations Rockall and Porcupine Seabight (Ireland), Mingulay (Scotland) and Skagerrak (Norway). As the success rate of the COI amplification in all specimens varied along the COI fragment for the M1M6 and I3M11 partitions (see CHAPTER V), we used the latter for these analyses. Haplotype (*h*) and nucleotide diversity (π), analysis of molecular variance (AMOVA) and population pairwise Φ_{ST} values using Tamura & Nei distances (Tamura and Nei, 1993) and 1000 permutations were calculated in Arlequin v 3.11 (Schneider et al., 2000). A Minimum Spanning Network was constructed in TCS v1.18 (Clement et al., 2000). Haplotypes were connected at the 95% confidence level and loops were resolved according to Crandall and Templeton (1993).

Results

Specimens belonging to the ESU H8 were distributed from the Southern deep-sea location of the Gulf of Cadiz, to the 'higher latitude' coral reef habitats in the Irish, the Scottish and the Norwegian sites. A total of 26 specimens from the three northern populations of H8 yielded five distinct COI I3M11 haplotypes containing 12 variable sites. The

geographical distribution of haplotypes is shown in Fig 1. Haplotype *H8 (ab*; corresponding to haplotypes H8a and H8b in the COI M1M6 partition, see CHAPTER V) was a widespread haplotype, shared between the Irish, the Scottish and the Norwegian population. Haplotype *H8(de)* was shared between the Irish and the Scottish populations (and was also found in the Gulf of Cadiz). Haplotypes *H8c* and *H8f* were private to the Irish margin, whereas *H8g* was private to the Scottish margin. Overall haplotype and nucleotide diversities were 0.5538 and 0.003988, respectively (Table 1). The deep-sea Irish and Scottish populations are clearly more diverse (haplotype diversity values of 0.5111 to 0.7778 and nucleotide diversity values of about 0.005) than the third location; the Norwegian population has null haplotype and genetic diversity values (Table 1). The Minimum Spanning Network indicated that the haplotypes *H8(ab), H8c*, and *H8(de)* were more closely related to each other than to the private haplotypes *H8f-g* (data not shown).

AMOVA did not detect any genetic structuring among the three populations (Φst=-0.00426, p= 0.542), and more than 99 % of the total variation was found within populations. The lack of genetic structure between populations was further confirmed by very low pairwise Fst values (-0.04661 to 0.01563, not significant, p=0.05).

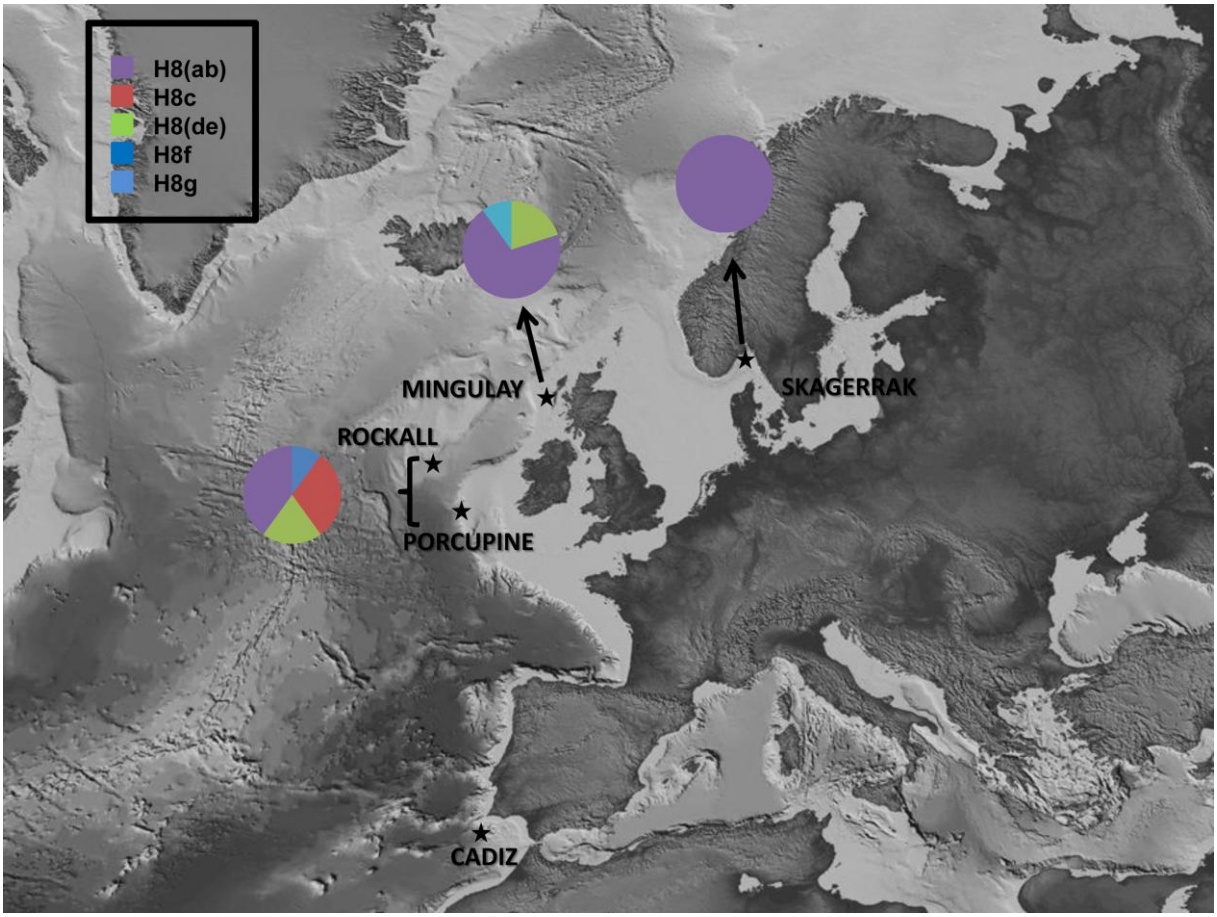

Fig 1. Map showing sampling locations of *Plocamionida microcionides* (H8 indicated by a star) and Northern geographical distribution of H8 mtDNA haplotypes. Map was provided by the project Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE).

Table 1. Haplotype and genetic diversity values within *Plocamionida microcionides*.

Population	N	Nh	Hd	π
ROCKALL+PORCUPINE	10	4	0.7778 (0.0907)	0.005078 (0.003688)
MINGULAY	10	3	0.5111 (0.1643)	0.005638 (0.004125)
SKAGERRAK	6	1	0	0
Overall 3 Populations	26	5	0.5538 (0.1028)	0.003988 (0.002791)

Population, number of individuals (N), number of haplotypes (Nh), haplotype diversity (Hd), and nucleotide diversity (π) are presented. Standard deviations for Hd and π are given in parenthesis.

Discussion

The deep-sea species *Plocamionida microcionides* showed a relatively high genetic variability between populations of Ireland, Scotland and Norway, with maximum uncorrected p-distance of 1.7 % in the I3M11 COI partition. In addition, the overall nucleotide diversity (π = 0.003988) and the number of haplotypes (five) identified across a distance of 800 to 2000

km were higher than for previous studies at similar or larger spatial scales using the M1M6 COI partition (e.g., π = 0.0006, Duran et al., 2004; π = 0.00049, Wörheide, 2006; π = 0.00058, Lopez-Legentil and Pawlik, 2009). They were of the same magnitude as the value found in recent intraspecific sponge studies using I3M11(π = 0.0039, Lopez-Legentil and Pawlik, 2009; π = 0.0042, Xavier et al., 2010b). It shows an informative genetic variation of the COI I3M11 partition and highlights the utility of this alternative COI partition for intraspecific analyses within *P. microcionides*.

The non-significant differentiation between the Northern populations of *P. microcionides* may suggest a certain genetic cohesion among deep-sea sponge populations. Studies on sponge phylogeography and population genetics in the Northeast Atlantic and the Mediterranean Sea are scarce, but the available data shows highly structured shallow-water populations as a result of restricted gene flow (Duran et al., 2004; Calderon et al., 2007; Blanquer et al., 2009; Xavier et al., 2010). Sponge larvae were indeed shown to be philopatric and recruited at short distance from the parental locations (Mariani et al., 2005; Mariani et al., 2006), and are therefore assumed to have limited dispersal abilities. The lack of significant genetic structure within *P. microcionides* from the Irish to the Norwegian margin is inconsistent with these characteristics and could reflect a lack of statistical resolution, due to the limited number of individuals. It could also be caused by the random evolutionary history of the locus analyzed, as selection and incomplete lineage sorting can affect patterns of genetic differentiation (Whitlock and McCauley, 1999). Assuming that the I3M11 partition reflects a certain gene flow among sponge populations, it raises questions about the possible longer planktonic period of some planktotrophic sponge larvae in the deep sea, which is a low energy (i.e., low food inputs) environment compared to shallow water (Gage and Tyler, 1991) . In general, the lack of genetic differentiation found here appears to be related with main ocean currents along the slope. De Mol et al., (2002; 2005) suggested that larvae of scleractinian corals took advantage of the currents created by the intrusion of the Mediterranean Outflow Water (MOW) into the North Atlantic in order to spread along the European margins. In addition, patches of CWC reefs were suggested to represent stepping-stone habitats for larval dispersal along the North East Atlantic margin (Reveillaud et al., 2008). Recent studies already suggest that the pattern of genetic structure within the sponge species *Xestospongia muta* is connected to patterns of ocean currents in the Caribbean (Lopez-Legentil and Pawlik, 2009). The fact that individuals from the Norwegian margin

grouped with individuals from the Irish and Scottish margin could be due to the colonization of migrants from southern populations to the Skagerrak area. This is in accordance with the moderate genetic differentiation reported among *Lophelia pertusa* offshore subpopulations along the European margin (Le Goff-Vitry and Rogers, 2005), and phylogenetic data on the coral associate sponge species *Hexadella dedritifera* (Reveillaud et al., 2010) between reefs from the NE Atlantic open slope. The observed higher genetic diversity at lower latitudes combined with the geographical distribution of COI haplotypes is then consistent with paleoclimate evidences for the Holocene age of 'high latitude' deep-water corals exposed at the seabed (e.g., along the Irish and Norwegian margin), while deep-water corals from lower latitudes show continuous growth over the last 53.5 ka (e.g., the Gulf of Cadiz, Schroder-Ritzrau et al., 2005; Wienberg et al., 2009). A current model of glacial refugia (Hewitt, 2004) supports genetically more diverse populations in the South and a lower diversity in locations that suffered the strongest anomalies during the Last Glacial Maximum (23.000-18 000 BP), such as the Norwegian Skagerrak population. Another separate marker, such as microsatellites or SNPs (Single Nucleotide Polymorphism), should however be studied to confirm this pattern. The single haplotype and low nucleotide diversity found in the Skagerrak area could also reflect a potential demographic bottleneck in recent time. This is however a process that would need to be confirmed by additional genes. Nevertheless, our study suggests the importance of deep-sea current patterns in a climate-changing environment and the study of life-history traits within sponge species in the deep-sea, which are of key importance to understand the geographic distribution of the phylum Porifera at great depths.

Conclusion

The I3M11 partition of the COI gene suggested a gene flow among the populations of *P. microcionides* associated with the 'high latitude' CWC reefs. Although these data constitute the first phylogeographic evidence for the connection of reef associated populations, these results are provisional and a more extensive sampling is necessary to support any firm statement.

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PUBLICATIONS

Chapters in this thesis:

Reveillaud J, Van Soest R, Derycke S, Picton B, Rigaux A, Vanreusel A (2011). Phylogenetic relationships among NE Atlantic Plocamionida Topsent (1927) (Porifera, Poecilosclerida): Under-Estimated Diversity in Reef Ecosystems**.** PLoS ONE 10.1371/journal.pone.0016533.

Reveillaud J, Remerie T, Van Soest R, Erpenbeck D, Cárdenas P, Derycke S, Xavier JR, Rigaux A, Vanreusel A (2010). Species boundaries and phylogenetic relationships between Atlanto-Mediterranean shallowwater and deep-sea coral associated Hexadella species (Porifera, Ianthellidae). Molecular Phylogenetics and Evolution 56 :104–114.

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Reveillaud J, Allewaert C, Pérez T, Vacelet J, Banaigs B, Vanreusel A. Relevance of an Integrative Approach for Taxonomic Revision in Sponge Taxa: Case Study of the Shallow-water Atlanto-Mediterranean Hexadella (Porifera, Ianthellidae). Manuscript in preparation.

Other publications:

Cárdenas P, Xavier JR, **Reveillaud J**, Schander C, Tore Rapp H (Accepted) Molecular Phylogeny of the Astrophorida (Porifera, Demospongiaep) Reveals an Unexpected High Level of Spicule Homoplasy. PLoS ONE. Journal.pone-D-10-04301R1.

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Noé SU, Lembke-Jene L, **Reveillaud J**, Freiwald A (2007). Growth banding and age determination of a unique fossil primnoid gorgonian skeleton (Octocorallia) from the Bay of Biscay. Facies 53 (2): 177-188.

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Molecular Evolution Workshop, MBL, Woods Hole, MS, USA (27/07-15/08/2008)
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'Access to genes and genomes with Ensembl', Ensembl workshop, Ghent University, Belgium (20/09/2007)
Coalescence Training Course, Natural Sciences Museum, Paris, France (21-25/11/2005)
Biodiversity and zoology of marine organisms Workshop, Roscoff Marine Station, France (01-27/08/2005)
Biodiversity, phylogeny and ecology of Porifera Training Course, Endoume Marine Station, Marseille, France (18-28/07/2005)
Geology and biology of cold-water coral reefs and carbonate mounds Workshop (EURODOM), Erlangen, Germany, (09-14/09/2004)

SYMPOSIA COMMUNICATIONS

2011 **J. Reveillaud**, L. Maignien, A. Vanreusel. Microbial communities analyses of sponges from shallow to deep-sea coral ecosystems**. 1st International Symposium on Sponge Microbiology**. Würzburg, Germany (21-22/03/2011) (Poster communication)

2010 **J. Reveillaud**, R. van Soest, S. Derycke, D. Erpenbeck, P. Cárdenas, J.R. Xavier, B. Picton, C. Goodwin, A. Rigaux, A. Vanreusel. Phylogenetic analyses of deep-water sponges associated with coral reefs in Europe provide new insights for their protection. **VIII Sponge Conference**. Girona, Spain (20-24/09/2010) (Oral Communication)

J. Reveillaud, A.Vanreusel**.** Phylogenetic and phylogeographic analyses of deep-water sponges associated with coral reefs in Europe. **Challenges in the study of deep-sea ecosystems' interconnectivity- Hermione Workshop.** Aveiro, Portugal (7-10/09/2010) (Oral Communication)

J. Reveillaud, R. van Soest, S. Derycke, D. Erpenbeck, P. Cárdenas, J.R. Xavier, B. Picton, A. Rigaux, A. Vanreusel. Plans to protect deep-water coral ecosystems along the European margins: new insights from phylogenetic analyses of the associated Porifera. **12th Deep-sea Symposium, Reikjavik, Iceland.** (7-11/06/2010) (Oral Communication)

2009 **J. Reveillaud**, P. Cárdenas, R. van Soest, A.Vanreusel. Biodiversity in European deep-water coral ecosystems: insights from barcoding to large-scale phylogenetic analyses of the associated sponges. **"Celebrating Darwin: From The Origin of Species to Deep Metazoan Phylogeny"**, the Humboldt University, Berlin, Germany (4-7/03/2009) (Poster presentation)

J. Reveillaud,T. Remerie, R. van Soest, A. Vanreusel. Plans to protect deep-water coral ecosystems: insights from large-scale phylogenetic analyses of the dominating Porifera. **Evolutionary islands 150 years after Darwin**. National Museum of Natural History in Leiden, the Netherlands (11-13/02/2009) (Poster presentation)

2008 **J. Reveillaud**, T. Remerie**,** R. van Soest, A. Vanreusel. Hidden Genetic diversity of sponges associated with cold-water coral ecosystems along the European margin. **Invited speak at the University of Bergen,** Norway (6/06/2008) (Oral communication)

J. Reveillaud, T. Remerie, R. van Soest, A. Vanreusel, J.P. Henriet. Genetic evidence for cryptic speciation in sponges associated with cold-water coral ecosystems. **10 years of Cold Water Coral Ecosystem and Carbonate Mound Research**, Het Pand, Ghent University, Belgium (17-18/01/2008) (Oral communication)

2007 **J. Reveillaud**, T. Remerie, R. van Soest, A. Vanreusel, J.P. Henriet From DNA barcoding to population genetics of broad range sponge taxa associated with cold-water coral reefs along the European margins. **DNA Barcoding in Europe**, EDIT, Leiden, Netherlands (3-5/10/2007) (Poster presentation)

2006 **J. Reveillaud**, T. Remerie, R. van Soest, A. Vanreusel, J.P. Henriet. Population genetics study of five broad range sponge taxa associated with cold-water coral reefs along the European margins, **International Sponge Conference**, Buzios, Brazil (7-13/05/2006) (Poster presentation)

2005 **J. Reveillaud**, J.C. Sorbe, J.P. Henriet. On the traces of forgotten deep-water coral reefs in the Bay of Biscay. **Earth Geosphere- Biosphere coupling meeting**, , Erlangen, Germany (24-29/09/2005) (Poster presentation)

2004 **J. Reveillaud**, J.P. Henriet. A GIS approach in the exploration and the conservation of deep-water coral habitats in the Gulf of Biscay. **EURODOM Mid Term Review meeting**, Erlangen, Germany (8/09/2004) (Oral communication)

2003 **J. Reveillaud**, E. Horjales, E. Rudino, A. Licea, R. Arreola. Structure model of the complexe Cn2-scFv chicken's clones neutralizing the Cn2 toxin from the Mexican scorpion Centruroides noxius Hoffman aimed to localize the epitope. **Biotechnologia Habana**, Habana, Cuba (23-28/11/2003) (Poster communication)

OCEANOGRAPHIC CAMPAIGNS

MEDECO, *R/V Pourquoi Pas?,* Santa Maria di Leuca, Italy (11-18/10/2007)
CADIPOR II, *R/V Belgica*, Gulf of Cadiz, off Larache, Morocco (17- 27/05/2005)
CADICOR (MD-138) *R/V Marion Dufresne*, Malta – Lisbon (28/05- 07/06/2004)

GRANTS

2010 **Fonds Wetenshappelijk Onderzoek (FWO):** Travel grant for attendance of the Deep-sea symposium, Reykjavik,Iceland

2008 **Synthesis grant:** Two-weeks visit to the Zoological Museum of Amsterdam, Netherlands

Fonds Wetenshappelijk Onderzoek (FWO): Travel grant for attendance of the Workshop on Molecular Evolution, MBL, Woods Hole, USA

Mohn Fund grant: Scholarship to support a barcoding/taxonomic collaborative work between Ghent University and Bergen University, Norway.

2006 **Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT):** PhD Fellowship

2004 **EU Marie Curie Research fellowship (EURODOM):** Two-years young scientist research/ scientific diffusion Fellowship

2001 **Chapell Hill University, NC, USA:** Travel grant for Student Exchange Program

STUDENTS' SUPERVISION

2010 Céline Allewaert- Erasmus Mundus Master in Marine Biodiversity and Conservation (EMBC), Ghent University. Project: Revision of *Hexadella pruvoti* and *Hexadella racovitzai* (1 year)
2009 Mascart Thibaud- 2nd master Marine and lacustrine sciences, Ghent University. Project: Professional internship: DIVA-Artabria II 2009 deep-sea campaign (2 weeks)
2009 Brecht Verhelst, Marteen van Cappellen, Korneel de Rynck-Bachelor students in Biology, Ghent University**.** Project: Assessing genetic variation of nuclear introns within the three deep-sea sponge species *Desmacella inornata*, *Poecillastra compressa*, *Plocamionida ambigua* (1 month)
2007 Hugo Lineart- MsC student in Biology, Ghent University. Project: Exploration of a deep-sea sponge kingdom; from their collection to their DNA information analysis, what sponges will tell us about cold-water coral reefs? (1 year)

KNOWLEDGE AND SKILLS

Bioinformatics: DNA sequence analysis (Seqman, ClustalW, MAFFT), public databases (EMBL, NCBI, Genebank, PDB), phylogenetic reconstruction (MEGA, (Mr)Modeltest, PAUP, MrBayes, RAxML, Forcon, Genedoc, Proseq, Treefinder, Treeview, Figtree), phylogeography (TCS, Arlequin)
IT languages: notions of Unix and Perl, introduction to BIOPERL, SQL, MYSQL
Laboratory work: DNA extraction, PCR and sequencing, gel electrophoresis, cloning, etc…
Mapping: GIS (ArcView)

LANGUAGES

French: mother tongue
English: read, spoken and written
Spanish: read, spoken and written
Dutch and German: good notions

OUTREACH ACTIVITY

Author of texts for the documentary ' Hidden Corals', Marenostrum Terranostra, Barcelona 2010, realized in collaboration with the Dr. Angelo Camerlengui and Dr. Ben de Mol (University of Barcelona, Spain).